NEW PERSPECTIVES ON THE BIOLOGY OF NECTARIES AND NECTARS

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NEW PERSPECTIVES ON THE BIOLOGY OF NECTARIES AND NECTARS

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Image: Massimo Nepi

The number of currently known, described and accepted plant species is ca 374,000, of which approximately 295,00 (79%) are angiosperms. Almost 90% of this huge number of flowering plants is pollinated by animals (mostly insects) via nectar-mediated interactions. Notably, three-fourths of the leading global crop plants produce nectar and are animal pollinated, which is estimated to account for one-third of human food resources. Nectar can also be produced on tissues outside of flowers, by so-called extrafloral nectaries, and commonly mediate interactions with 'body-guard' ants and other pugnacious insects that defend the plant from herbivores. Extrafloral nectar is present in almost 4,000 plant species, a majority of them in the angiosperms. This brief summary on the occurrence of nectar in the plant kingdom is just to highlight that nectar has a fundamental role in two basal functions that allow the maintenance of our ecosystems: sexual plant reproduction and protection of plants from herbivory. Despite playing essential ecological and evolutionary functions, our current knowledge about nectar is largely incomplete;

however, new research directions and perspectives on nectaries and nectars have arisen in recent years.

In the last two decades, there were only a few 'moments' in which nectar was the main character in international meetings or in published books. In 2002, the first (and only) international meeting "Nectar and nectary: from biology to biotechnology" dedicated exclusively to nectar and nectaries was held in Italy (Montalcino, Siena) and in 2003 the proceedings were published in a special volume of Plant Systematics and Evolution (238, issue 1-4). In 2007, the book Nectar and Nectaries was published (Springer) with most of the contributions provided by authors that attended the meeting in Italy. Another book dedicated to nectar was published in 2015 (Nectar: Production, Chemical Composition and Benefits to Animals and Plants, Nova Science Publishers) covering aspects mainly related to nectar chemical composition and plant-pollinator interactions. Similarly, symposia focused on nectar have been organized within the International Botanical Congress in 2011 and 2017.

Considering that the last few years has yielded essential developments in the understanding of nectar biology, we thought now is the moment to further stimulate research on this important topic. This aim has been met through 18 papers published in our Research Topic New Perspectives on the Biology of Nectaries and Nectars, with subjects spanning evolution and ecology to nectar chemistry and nectary structure.

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NECTAR AND CLIMATE CHANGE

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Common Features Between the Proteomes of Floral and Extrafloral Nectar From the Castor Plant (*Ricinus Communis*) and the Proteomes of Exudates From Carnivorous Plants

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Label-free quantitative proteome analysis of extrafloral (EFN) and floral nectar (FN) from castor (*Ricinus communis*) plants resulted in the identification of 72 and 37 proteins, respectively. Thirty proteins were differentially accumulated between EFN and FN, and 24 of these were more abundant in the EFN. In addition to proteins involved in maintaining the nectar pathogen free such as chitinases and glucan 1,3-beta-glucosidase, both proteomes share an array of peptidases, lipases, carbohydrases, and nucleases. A total of 39 of the identified proteins, comprising different classes of hydrolases, were found to have biochemical matching partners in the exudates of at least five genera of carnivorous plants, indicating the EFN and FN possess a potential to digest biological material from microbial, animal or plant origin equivalent to the exudates of carnivorous plants.

Keywords: floral nectar, extrafloral nectar, carnivorous plants, *Ricinus communis*, proteomics

INTRODUCTION

Nectar is an energy rich substance secreted by glands situated at the base of flowers (floral nectar, FN) or in other parts such as leaves, stems, rachis, etc. (extrafloral nectar, EFN) (Shah et al., 2016). While FN attracts pollinating insects, EFN attracts aggressive ants and other mutualists, which in turn provide antiherbivore protection (Marazzi et al., 2013). Although these functional aspects are widely recognized (Roy et al., 2017), the dynamic of the relation FN/pollinators and EFN/mutualists is poorly known, especially the biochemical properties of the nectar which play a role in the attraction of particular pollinator/mutualists. Additionally, little is also known about the biochemical machinery involved in the secretion of nectar (Heil, 2015), and even less on the proteins responsible for maintaining these carbohydrate rich energy sources free of pathogens (Park and Thornburg, 2009; Heil, 2015; Roy et al., 2017). Up to now, only a limited number of studies have presented data on the proteomes of EFN and FN (Orona-Tamayo et al., 2013; Seo et al., 2013; Zhou et al., 2016). These studies demonstrated the worth of establishing the complete proteomes of EFN and FN to acquire a better understanding of the preference of certain mutualists for a particular

type of EFN or FN and paved the way for establishing that proteins in nectars have roles which go beyond helping in keeping the nectar pathogen free.

The notion that the proteome of EFN is larger than FN is well established (Coulter et al., 2012) but it has not yet been tested directly. Likewise, it is still not known whether nectars from different sources possess a common set of proteins with the general role of keeping it pathogen free and a variable number of proteins conferring to a certain EFN or FN properties underlying its acceptance/rejection by mutualist animals. Last of all, the possibility that EFN and FN are involved in aspects of plant biology other than pollination and defense have not been investigated so far.

In order to address these questions, we have performed a label-free quantitative proteome analysis of EFN and FN from the castor plant (*Ricinus communis*) using nectar collected from plants grown under similar temporal and environmental conditions. Our analysis provides evidence for the presence in EFN and FN of a wide array of hydrolases (peptidases, carbohydrases, lipases, and nucleases) and a number of proteins related to the dismantling of the cell wall of plants and fungi. Additionally, we show that a sizable fraction of the proteins from EFN and FN have counterparts in the proteomes of the exudates of carnivorous plants.

MATERIALS AND METHODS

Acquisition of Floral (FN) and Extrafloral (EFN) Nectar

Plants were grown under irrigation, in the experimental field of the Agronomy School, Federal University of Ceará, Fortaleza, Brazil. Nectar collection was performed daily from 6 to 8 a.m. (1–3 h after sunrise), by the use of a handmade glass syringe, totalizing four and three biological samples for the FN and EFN, respectively. The material collected was immediately centrifuged (10,000 g), and sterile-filtered and kept at −20°C until used.

Protein Precipitation and Trypsin Digestion

Collected FN and EFN were submitted to protein precipitation using cold acetone with 10% TCA as described (Vasconcelos et al., 2005). Precipitated proteins from both FN and EFN were solubilized in 7 M urea, 2 M thiourea. An aliquot was used to determine protein concentration by the Qubit Protein Assay Kit (Qubit® 2.0 Fluorometer, Thermo Scientific) according to the manufacturer's instructions. For protein digestion, 50 μg of proteins of each sample was reduced with dithiothreitol at a final concentration of 10 mM for 1 h at 30°C, followed by iodoacetamide alkylation at 40 mM final concentration for 30 min at room temperature in the dark. Samples were diluted with 50 mM ammonium bicarbonate to 1 M urea concentration and after trypsin addition (1:50, w/w, Sequencing Grade Modified Trypsin, V5111, Promega), solutions were incubated at 35°C for 18 h. Tryptic hydrolysis was stopped with TFA at 0.1% final concentration. After digestion peptides were concentrated and desalted by custom-made chromatographic Poros 50 R2 (PerSeptive Biosystems) reverse phase tip-columns and dried on vacuum concentrator (Thermo Scientific) (Gobom et al., 1999).

nLC-MS Analysis

Peptides resuspended in 0.1% formic acid were quantified by the Qubit Protein Assay Kit. MS analysis was performed in triplicates for each biological replicate from FN and EFN samples in a nano-LC EASY-II coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Two μg of peptides were loaded in a precolumn (2 cm length, 100 μm I.D., packed in-house with ReproSil-Pur C18-AQ 5 μm resin–Dr. Maisch GmbH HPLC) and fractionated in a New Objective PicoFrit® Column (25 cm length, 75 μm I.D., packed in-house with ReproSil-Pur C18-AQ 3 μm resin–Dr. Maisch GmbH HPLC). Peptides were eluted using a gradient from 95% phase A (0.1% formic acid, 5% acetonitrile) to 40% phase B (0.1% formic acid, 95% acetonitrile) for 107 min, 40–95% phase B for 5 min and 95% B for 8 min (total of 120 min at a flow rate of 200 nL/min). After each run, the column was washed with phase B and re-equilibrated with phase A. m/z spectra were acquired in a positive mode applying data-dependent automatic MS and MS/MS acquisition. MS scans (m/z 350–2,000) in the Orbitrap mass analyzer at resolution 30,000 (at m/z 400), 1×10^6 AGC and 500 ms maximum ion injection time, were followed by HCD MS/MS of the 10 most intense multiply charged ions in the Orbitrap at 10,000 signal threshold, resolution 7,500 (at m/z 400), 50,000 AGC, 300 ms maximum ion injection time, m/z 2.5 isolation width, 10 ms activation time at 30 normalized collision energy and dynamic exclusion enabled for 30 s with a repeat count of 1.

Database Search

Raw data were inspected in Xcalibur v.2.1 (Thermo Scientific). Database searches were performed using Proteome Discoverer 2.1 (Thermo Scientific) using Sequest™ algorithm against *Ricinus communis* database downloaded from Uniprot database March 2017. The searches were performed with the following parameters: MS accuracy 10 ppm, MS/MS accuracy 0.1 Da, trypsin digestion with two missed cleavage allowed, fixed carbamidomethyl modification of cysteine and variable modification of oxidized methionine and acetyl at protein N-terminus. Protein groups and, numbers of peptides were estimated using Proteome Discoverer using false discovery rates around 1% at protein and peptide level and peptide rank. Three technical replicates were obtained for each FN and EFN samples, constituted by four and three biological replicates, respectively. Proteins were considered identified when present in at least two technical replicates for each biological replicate and in at least two biological replicates for each nectar sample. Proteins were filtered by FDR less than 1% and the presence of at least one unique peptide.

Data Analysis

Quantification was estimated using the workflow node Precursor Ions Area Detector in Proteome Discoverer. The peak area average of the most abundant distinct peptides of each protein was used for its relative quantification. Proteins with peak area averages present in at least two technical and two biological replicates were used to generate the list of proteins quantified. Normalization was executed using the total peptide

amount where the total sum of the abundance values over all peptides identified within a file is used to correct the abundance in all files. Afterward, the values for the FN and EFN runs were merged and the total median was determined. A ratio of each protein between FN and EFN samples was measured and a *t*-test was performed to evaluate significant differences.

Proteins identified with the database description unclear or as "putative uncharacterized protein" were submitted to manual Blastp in Uniprot (http://www.uniprot.org/blast/) and NCBI (https://blast.ncbi.nlm.nih.gov) websites. Proteins with high identity were selected for the identification of uncharacterized proteins. The subcellular localization was predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2000) and the Top Hit Domains present in the identified proteins was evaluated by PFAM Batch sequence search (https://pfam.xfam.org/search).

RESULTS

Label-free quantitative proteomics was employed to characterize the proteins present in the extrafloral (EFN) and floral nectars (FN) of castor plants (*Ricinus communis*). The proteomes of EFN and FN are populated by 72 and 37 proteins, respectively (**Table 1**, Table S1). For FN, 19, 11 and seven proteins were present in two, three, and four biological replicates, respectively. In the EFN, 62 and 10 proteins appeared in two and three biological replicates, respectively. From these, 30 are shared by both nectar types while 7 and 42 are restricted to FN and EFN respectively. As assessed by the TargetP software, 70% of the identified proteins have an N-terminal signal peptide for the secretory pathway (Table S1). Among the proteins unique to the EFN, 20 of them have biochemical counterparts in the FN (**Table 1**); however, the EFN proteome has a greater complexity in terms of diversity of kinds of enzymatic activities. Beta-fructofuranosidase (B9R9R9) an enzyme known to balance sucrose levels in the extrafloral nectar of several species is among the proteins unique to the EFN.

Most of the proteins identified in this study are known to possess defined biochemical activity and/or physiological function in plants. Apart from 15 proteins, the remaining 64 can be tentatively sorted into the seven functional classes as shown in **Table 1**. Although a sizeable fraction of these proteins was previously identified in EFN and/or FN from other species, enzymes related to the dismantling of the cell wall (four pectinesterases, two polygalacturonases and one polygalacturonase inhibitor), protein hydrolysis (two xylem serine proteinases and one carboxypeptidases) have not been previously identified in any type of nectar.

The limited availability of the complete proteome of nectar from different species precludes a more precise appraisal regarding the distribution of these seven classes of proteins into EFN and FN of other plant taxa. However, as seen in **Table 1**, 39 out of the 79 proteins listed have identical predicted biochemical activities (biochemical matching partner) in the exudates of five genera of carnivorous plants. Apart from the proteins involved in defense functions, most of the other enzymes display hydrolytic activity against proteins, chitin, carbohydrates, lipids and nucleic acids as well as the capability of hydrolyzing/modifying components of the cell wall of plants or fungi. The presence of these enzymes provides evidence that the EFN and FN possess the enzymatic machinery to promptly digest biological material of microbial, plant or animal origin which happens to land into the floral or extrafloral nectaries.

Floral and extrafloral nectars are thought to possess a set of proteins that constitute the Carter-Thornburg nectar redox cycle, whose concerted action protect the nectar from infection (Liu and Thornburg, 2012). As **Table 2** shows, only the EFN has the complete set of proteins of the Carter-Thornburg nectar redox cycle, while in FN only one enzyme (B9SAZ8) from this cycle could be identified, thus probably indicating that FN has alternative modes to avoid microbial infection. Both EFN and FN share a carbonic anhydrase (**Table 1**), that may act to avoid abrupt changes in the nectar pH, thus stabilizing the different enzymatic activities (see **Table 2**) in the nectar.

Thirty proteins were differentially accumulated between EFN and FN (Table S1), and from these, 23 were distributed among all the seven functional classes shown in **Table 1**, while seven were classified as proteins of unknown function. Of the differentially expressed proteins, a total of 24 were more abundant in the EFN. A desiccation-related protein (B9T0V6) displayed the highest rate of differential expression, followed by a carbonic anhydrase B9T346 and a glucan 1,3-beta-glucosidase (B9RJG5), with a fold change of 17.4, 14.4, and 10.8 respectively. As discussed below, the functional significance of the differential expression of these proteins may bear relation to the persistent nature of the extrafloral nectary as compared to the floral nectary.

DISCUSSION

We present here a direct comparison between the proteomes of EFN and FN from the same species, collected under similar temporal and environmental conditions. It confirms the greater complexity of the EFN, both in number of proteins species and in terms of biochemical capability, which probably underlies functional differences between the two nectar types.

The mechanisms employed to create an environment hostile to microbial infestation is a moot point in nectar biology (González-Teuber et al., 2009; Park and Thornburg, 2009; Heil, 2015; Roy et al., 2017). The task of creating an environment antagonistic to microbial infestation through the production of hydrogen peroxide seems to be one of the chosen strategies in EFN, as indicated by the presence in its proteome of a full set of proteins from the Carter-Thornburg redox-cycle (**Table 2**; Carter and Thornburg, 2004). The absence of these proteins in FN shows that rather than to rely on the steady production of hydrogen peroxide, the FN counts with a wide array of hydrolases, which may act in concert to ward off microbial growth. It should also be noted that in EFN the

TABLE 1 | Functional classes of proteins identified in EFN and FN proteomes from castor plants (*Ricinus communis*), and the genera of carnivorous plants in which counterpart proteins were identified.

Sample	Accession	Description	Genus	References
PEPTIDASES				
EFN	B9T719	Aspartic proteinase nepenthesin-1, putative	Dioneae, Nepenthes, Cephalotus, Sarracenia	Hatano and Hamada, 2008; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
EFN	B9RNR8	Aspartic proteinase nepenthesin-2, putative	Dioneae, Nepenthes, Cephalotus, Sarracenia	Hatano and Hamada, 2008; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
EFN	B9SNP5	Carboxypeptidase	Dioneae	Schulze et al., 2012; Bemm et al., 2016; Lee et al., 2016; Rottloff et al., 2016
EFN	B9S815	Serine carboxypeptidase, putative	Dioneae, Nepenthes	Schulze et al., 2012; Bemm et al., 2016; Lee et al., 2016; Rottloff et al., 2016
EFN	B9T4J8	Xylem serine proteinase 1, putative	–	–
EFN	B9R726	Xylem serine proteinase 1, putative	–	–
FN/EFN	B9RNR9	Aspartic proteinase nepenthesin-2, putative	Dioneae, Nepenthes, Cephalotus	Hatano and Hamada, 2008; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
FN/EFN	B9T568	Serine carboxypeptidase, putative	Dioneae, Nepenthes	Schulze et al., 2012; Bemm et al., 2016; Lee et al., 2016; Rottloff et al., 2016
CHITINASES				
EFN	B9S6S0	Class I chitinase, putative	Dioneae, Nepenthes, Cephalotus, Drosera	Hatano and Hamada, 2012; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
EFN	B9T8H9	Class IV chitinase, putative	Dioneae, Nepenthes, Cephalotus, Drosera	Hatano and Hamada, 2012; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
EFN	B9RIP3	Hevamine-A, putative	Dioneae, Nepenthes, Cephalotus, Drosera	Hatano and Hamada, 2012; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
FN/EFN	B9SBZ8	Chitinase, putative	Dioneae, Nepenthes, Cephalotus, Drosera	Hatano and Hamada, 2012; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
EFN	B9RIP2	Acidic endochitinase SE2, putative	Dioneae, Nepenthes, Cephalotus, Drosera	Hatano and Hamada, 2012; Schulze et al., 2012; Lee et al., 2016; Rottloff et al., 2016
LIPASES				
FN	B9SJ71	Hydrolase, acting on ester bonds, putative (*phospholipase C2*)	Dioneae, Nepenthes	Schulze et al., 2012;
EFN	B9SQQ6	Zinc finger protein, putative (*gdsl esterase/lipase*)	Dioneae, Nepenthes	Schulze et al., 2012; Rottloff et al., 2016
EFN	B9RM21	Zinc finger protein, putative (*gdsl esterase/lipase*)	Dioneae, Nepenthes	Schulze et al., 2012; Rottloff et al., 2016
FN/EFN	B9T8L6	Zinc finger protein, putative (*gdsl esterase/lipase*)	Dioneae, Nepenthes	Schulze et al., 2012; Rottloff et al., 2016
NUCLEIC ACID HYDROLYSIS				
FN/EFN	B9SZ66	Wound-induced protein WIN1 (*pathogenesis-related protein 4*)	Dioneae, Nepenthes, Cephalotus	Schulze et al., 2012; Bemm et al., 2016; Lee et al., 2016
FN/EFN	B9SZ67	Wound-induced protein WIN1 (*pathogenesis-related protein 4*)	Dioneae, Nepenthes, Cephalotus	Schulze et al., 2012; Bemm et al., 2016; Lee et al., 2016
CELL WALL MODIFYING ENZYMES				
EFN	B9T7M3	Alpha-glucosidase, putative	Nepenthes	Rottloff et al., 2016
EFN	B9RTU8	Basic 7S globulin 2 small subunit (*xylanase inhibitor*)	–	–

(Continued)

TABLE 1 | Continued

Sample	Accession	Description	Genus	References
EFN	B9RIY8	Beta-glucosidase, putative	Nepenthes	Rottloff et al., 2016
EFN	B9S377	Ceramidase, putative	–	–
EFN	B9RYU9	Endoglucanase	Nepenthes, Dionaea, Cephalotus, Drosera, Sarracenia	Hatano and Hamada, 2012; Schulze et al., 2012; Bemm et al., 2016; Lee et al., 2016
EFN	B9T103	Glucan endo-1,3-beta-glucosidase, putative	Nepenthes, Drosera	Rottloff et al., 2016
EFN	B9SU04	Glucan endo-1,3-beta-glucosidase, putative	Nepenthes, Drosera	Rottloff et al., 2016
EFN	B9SAU3	Pectinesterase	–	–
EFN	B9RD90	Pectinesterase	–	–
EFN	B9RFP1	Polygalacturonase, putative	Dioneae, Nepenthes	Schulze et al., 2012
EFN	B9S447	Putative uncharacterized protein (*xylanase inhibitor*)	–	–
EFN	B9T2C7	Serine-threonine protein kinase (*polygalacturonase inhibitor*)	Dionaea, Nepenthes	Rottloff et al., 2016
FN/EFN	B9T6M9	Glucan endo-1,3-beta-glucosidase, basic isoform, putative	Nepenthes	Rottloff et al., 2016
FN/EFN	B9RBE5	Glucan endo-1,3-beta-glucosidase, putative	Nepenthes	Rottloff et al., 2016
FN/EFN	B9SMA9	Laccase	–	–
FN/EFN	B9RU20	Pectinesterase	–	–
FN/EFN	B9RA18	Pectinesterase	–	–
EFN	B9T3Q0	Non-specific lipid-transfer protein	Dioneae, Nepenthes, Drosera	Schulze et al., 2012; Bemm et al., 2016; Rottloff et al., 2016
EFN	B9SRS0	Non-specific lipid-transfer protein	Dioneae, Nepenthes, Drosera	Schulze et al., 2012; Bemm et al., 2016; Rottloff et al., 2016
EFN	B9SE97	Peroxidase	Dioneae, Nepenthes, Cephalotus	Hatano and Hamada, 2012; Bemm et al., 2016; Lee et al., 2016; Rottloff et al., 2016
EFN	B9R8I7	Multicopper oxidase, putative	–	–
FN/EFN	B9RCG6	Polygalacturonase, putative	Dioneae	Schulze et al., 2012
FN/EFN	B9RJG5	Putative uncharacterized protein (*probable glucan 1,3-beta-glucosidase A*)	Dioneae, Nepenthes	Rottloff et al., 2016
FN/EFN	B9RBC9	Structural constituent of cell wall, putative	–	–
FN	B9SBL2	Multicopper oxidase, putative	–	–
FN/EFN	B9S4B6	Peroxidase	Dionaea, Nepenthes	Hatano and Hamada, 2012; Bemm et al., 2016; Lee et al., 2016; Rottloff et al., 2016
EFN	B9S9S6	Putative uncharacterized protein (*fasciclin-like arabinogalactan protein 1*)	–	–
FUNCTION IN DEFENSE				
EFN	B9RC64	Osmotin, putative	Dioneae	Schulze et al., 2012; Bemm et al., 2016; Rottloff et al., 2016
FN/EFN	B9RC65	Osmotin, putative	Dioneae	Schulze et al., 2012; Bemm et al., 2016; Rottloff et al., 2016
EFN	B9T6Y3	Monodehydroascorbate reductase, putative	–	–
EFN	B9REW9	Superoxide dismutase [Cu-Zn]	Dionaea	Schulze et al., 2012
FN/EFN	B9SAZ8	Reticuline oxidase, putative	–	–
EFN	B9SAZ6	Reticuline oxidase, putative	–	–

(Continued)

TABLE 1 | Continued

Sample	Accession	Description	Genus	References
EFN	B9SB02	Reticuline oxidase, putative	–	–
EFN	B9T0V5	Putative uncharacterized protein (*desiccation-related protein*)	Sarracenia	Fukushima et al., 2017
FN/EFN	B9T0V6	Putative uncharacterized protein (*desiccation-related protein*)	Sarracenia	Fukushima et al., 2017
FN/EFN	B9T346	Carbonic anhydrase, putative	–	–
FN/EFN	B9RC10	Glucose-methanol-choline (Gmc) oxidoreductase, putative	–	–
FN/EFN	B9RGE3	Disease resistance protein RPM1, putative	–	–
EFN	B9S7U9	STS14 protein (*pathogenesis related protein PR-1*)	Dionaea	Schulze et al., 2012
CARBOHYDRATE METABOLISM				
FN	B9RG09	Transaldolase, putative		
EFN	B9R9R9	Beta-fructofuranosidase, soluble isoenzyme I, putative	–	–
EFN	B9SRG1	Enolase, putative	Nepenthes	Lee et al., 2016
FN/EFN	B9RAL0	Glyceraldehyde-3-phosphate dehydrogenase	–	–
FN/EFN	B9SP64	Phosphoglucomutase, putative	–	–
UNKNOWN FUNCTION				
FN	B9RQ33	5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase, putative	–	–
FN	B9S0I6	DNA binding protein, putative	–	–
FN	B9SKK5	Nucleoside diphosphate kinase	–	–
FN	B9SCN6	Putative uncharacterized protein	–	–
FN/EFN	B9RWF4	Elongation factor 1-alpha	–	–
EFN	B9RJM9	Putative uncharacterized protein	–	–
EFN	B9RK70	Putative uncharacterized protein	–	–
EFN	B9RNV2	Early nodulin 55-2, putative	–	–
FN/EFN	B9RPP7	DUF26 domain-containing protein 2, putative	–	–
FN/EFN	B9RS28	Mta/sah nucleosidase, putative	–	–
FN/EFN	B9RZI8	Alpha/beta hydrolase, putative	–	–
FN/EFN	B9S225	Mta/sah nucleosidase, putative	–	–
FN/EFN	B9SXP3	Putative uncharacterized protein	–	–
FN/EFN	B9T494	Auxin-induced in root cultures protein 12, putative	–	–
EFN	B9REF0	Hydrolase, hydrolyzing O-glycosyl compounds, putative	–	–

array of hydrolases is wider than in FN, indicating that EFN may rely on at least two different strategies to avoid microbial infection.

One of the most conspicuous evidence for the functional distinction between EFN and FN is the absence in the FN of a beta-fructofuranosidase. This enzyme is known to adjust the carbohydrate composition of the extrafloral nectar to exclude non-mutualistic ants (Heil et al., 2005; González-Teuber et al., 2009). The quantitative analysis we performed (Table S1), provides further support for the biochemical and functional differences between EFN and FN. Most of the differentially expressed proteins were more abundant in the EFN. A desiccation-related protein (B9T0V6), a carbonic anhydrase (B9T346) and a glucan 1, 3-beta-glucosidase (B9RJG5) were the most abundantly expressed. The desiccation-related proteins are involved in promoting the tolerance of plants to desiccation, although an alternative role as defense proteins against microorganisms has been proposed (Zha et al., 2013). Its differential accumulation in the EFN may be causally related to the long period of time in which the extrafloral nectary is metabolically active. The nectar produced at a given period if not consumed is either evaporated or reabsorbed,

TABLE 2 | The Carter-Thornburg redox cycle enzymes identified in the FN and EFN of castor plants (*Ricinus communis*).

EFN	B9REW9	Superoxide dismutase [Cu-Zn]
EFN/FN	B9T346	Carbonic anhydrase, putative
EFN	B9T6Y3	Monodehydroascorbate reductase, putative
EFN	B9RTU8	xyloglucan-specific endo-β-1,4-glucanase inhibitor
EFN	B9S447	xyloglucan-specific endo-β-1,4-glucanase inhibitor
EFN/FN	B9SAZ8	Reticuline oxidase, putative
EFN	B9SAZ6	Reticuline oxidase, putative
EFN	B9SB02	Reticuline oxidase

leading to the periodical increase in the osmotic pressure of the surface of the extrafloral nectary so that the presence of this desiccation related protein would counterbalance the detrimental biological effect of a high osmotic pressure. Carbonic anhydrase is a metalloenzyme that catalyzes the interconversion of CO_2 and HCO_3^- and it is suggested to have a role in the stabilization of nectar pH (Park and Thornburg, 2009), thus propitiating the maintenance of the biochemical and functional properties of the nectar proteins; buffering nectar to a physiological pH would be essential if the enzymes in the nectar are to remain active. Finally, glucan 1, 3-beta-glucosidase (B9RJG5) has asserted roles in plant development and hold a well-characterized activity against phytopathogenic fungi (Balasubramanian et al., 2012). Again, the long-lasting nature of the extrafloral nectary as compared to the floral nectary provides a reason for the differential abundance of this protein in the EFN.

The wide array of peptidases, nucleases, lipases, cell wall modifying enzymes and chitinases found in the proteomes of EFN and FN, raises qualms about the contention that action of nectar proteins is limited to prevent microbial growth in the nectar. Apart from few proteins, notably the pectinesterases and polygalacturonases, most of the proteins identified in the proteomes of EFN and FN were previously identified in varied biochemical analysis of nectars from the castor plant and from other sources (see for example: Harper et al., 2010; Orona-Tamayo et al., 2013; Seo et al., 2013; Millán-Cañongo et al., 2014; Zha et al., 2016; Zhou et al., 2016). However, as these studies were generally focused in the identification of proteins that could have a role in maintaining the nectar a pathogen-free environment, the significance of proteins other than the classical pathogen-related proteins, was not reckoned worth of further inquiry. Our proteome analysis support to the idea that one of the roles of the nectar proteins is to prevent microbial growth, keep the nectar pH at the physiological level and provide a pH-balanced meal for visitors (Park and Thornburg, 2009). Although some of the identified proteins are known to be involved in defense reaction, most of the others cannot possibly be involved either in pathogen control or pH maintenance and therefore the adaptive role of these proteins is a question that warrants investigation.

The widely held notion that nectar represents phloem sap, does not find support in the data we present here. The proteomes of FN and EFN have not much in common with the proteomes of phloem, both in terms of number and diversity of functions of the proteins (for reviews see Carella et al., 2016; Rodríguez-Celma et al., 2016). In this context, it is relevant to point out that studies dealing with the transcriptome (Doering-Saad et al., 2006) and proteome (Barnes et al., 2004) of the phloem sap of *R. communis* indicated a much higher number and diversity of proteins than that we found in our proteome analysis of FN and EFN of *R. communis*.

As shown in **Table 1**, the proteomes of EFN and FN share a number of peptidases, lipases, nucleases, carbohydrases, and chitinases with the exudates of carnivorous plants. These hydrolytic enzymes act to give to the exudates the capability of digesting any prey that happens to be trapped, thus making available to the host plant sources of nitrogen, phosphorous, carbon, etc. (Ellison and Gotelli, 2009; Fukushima et al., 2017; Thorogood et al., 2017). Also shared by EFN, FN and the exudates are the proteins whose activity creates a pathogen-free environment, notably glucanases and chitinases. The identification of pectinesterases and polygalacturonases both in EFN and FN, point out a heightened potential for digesting complex carbohydrates of plant origin, including the major constituents of the cell wall. It thus appears to be likely that any biological material landing in the floral or extrafloral nectaries are liable to be digested, resulting in the production of nitrogen and carbon sources, which may be absorbed by the nectary gland and distributed throughout the plant to provide additional nutrition. This hypothesis begs for a careful experimental testing.

It is usually claimed that carnivory has evolved independently at least six times in five angiosperm orders and seems to be restricted to 0.2% of plant species (Ellison and Gotelli, 2009). However, following the report of a hitherto unknown type of herbivory in underground leaves from three *Philcoxia* species (Pereira et al., 2012), the authors suggested that carnivory may not be a rare trait and that the number of carnivorous plants is underestimated, thus giving support to a notion expressed years before by Chase et al. (2009), which famously claimed that "we are surrounded by murderous plants." Therefore, whether carnivory is a pervasive trait continues to be a contentious issue, but the common features shown here between the proteomes of EFN and FN of castor plants and the proteome of exudates from carnivorous plants, adds a new twist to this debate: as a result of nectar secretion, extrafloral and floral nectaries are competent to digest biological material from animal or plant origin which land on its surface. Considering that these glands are widespread in the angiosperms and that these proteome features may be shared many other nectars, one is compelled to propose that we are indeed surrounded by "murderous plants." Whether the hydrolytic capabilities of EFN and FN has any adaptive value and whether the carbon and nitrogen sources generated are absorbed and systemically distributed throughout the plants, are issues entreating cautious experimentation.

Mass spectrometry raw data files are available at: PRIDE Archive (https://www.ebi.ac.uk/pride/archive/) project accession PXD009104.

AUTHOR CONTRIBUTIONS

FN and FC conceived and designed research. FN, AF, and FT conducted sample preparation and proteomics experiments. FN, AF, GD, and FC analyzed data. FN, AF, GD, and FC wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00549/full#supplementary-material

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Sex-Dependent Variation of Pumpkin (*Cucurbita maxima* cv. Big Max) Nectar and Nectaries as Determined by Proteomics and Metabolomics

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Nectar is a floral reward that sustains mutualisms with pollinators, which in turn, improves fruit set. While it is known that nectar is a chemically complex solution, extensive identification and quantification of this complexity has been lacking. *Cucurbita maxima* cv. Big Max, like many cucurbits, is monoecious with separate male and female flowers. Attraction of bees to the flowers through the reward of nectar is essential for reproductive success in this economically valuable crop. In this study, the sex-dependent variation in composition of male and female nectar and the nectaries were defined using a combination of GC-MS based metabolomics and LC-MS/MS based proteomics. Metabolomics analysis of nectar detected 88 metabolites, of which 40 were positively identified, and includes sugars, sugar alcohols, aromatics, diols, organic acids, and amino acids. There are differences in 29 metabolites between male and female nectar. The nectar proteome consists of 45 proteins, of which 70% overlap between nectar types. Only two proteins are unique to female nectar, and 10 are specific to male nectar. The nectary proteome data, accessible at ProteomeXchange with identifier PXD009810, contained 339 identifiable proteins, 71% of which were descriptively annotatable by homology to Plantae. The abundance of 45 proteins differs significantly between male and female nectaries, as determined by iTRAQ labeling. This rich dataset significantly expands the known complexity of nectar composition, supports the hypothesis of H+-driven nectar solute export, and provides genetic and chemical targets to understand plant–pollinator interactions.

Keywords: metabolomics, proteomics, nectar, *Cucurbita*, pumpkin, floral sex

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INTRODUCTION

Nectar is the most common floral reward used by angiosperms to mediate a mutualistic relationship with pollinators, and improves the plant's reproductive success by promoting outcrossing (Mitchell et al., 2009). In crops such as oilseed rape (Carruthers et al., 2017), sunflower (Mallinger and Prasifka, 2017), and pumpkin (Nepi and Pacini, 1993), variations in nectar composition and volume directly influence the frequency of pollinator visitation. Because 87 out

of 115 global food crops are dependent on or achieve improved fruit set through animal-mediated pollination (Klein et al., 2007), a potential future breeding goal could target improved nectar traits. However, in order to exploit this trait, a more comprehensive understanding of nectar composition is needed.

Nectar is a complex solution that, depending on the species, may contain some or all of the following constituents: carbohydrates, amino acids, vitamins, alkaloids, phenolics, terpenoids, lipids, metal ions, hormones, and proteins (Richardson et al., 2015; Roy et al., 2017). The two most predominant classes of metabolites are carbohydrates followed by amino acids (Lüttge, 1977). A system of nectar classification based on the ratios of predominant sugars proposed by Baker and Baker (1983) defines four classes of nectar: hexose-dominant, hexose-rich, sucrose-dominant, and sucrose-rich. Different clades of animals are attracted to different hexose-sucrose ratios and nectar amino acid profiles (Baker and Baker, 1983; Gardener and Gillman, 2002; Hendriksma et al., 2014; Nepi, 2014). Thus, nectar ecology studies typically define nectar composition based upon targeted analyses of predominant sugars and occasionally the amino acids. To date, few studies have applied metabolomics techniques to study nectar composition (Kram et al., 2008; Bender et al., 2012, 2013; Noutsos et al., 2015). Metabolomics, as used in this study, can potentially detect novel secondary metabolites important for pollinator attraction and health, which are instrumental in sustaining the ecosystem service of pollination.

While most analyses have concentrated on small molecular weight compounds, such as sugars, recent studies have revealed an abundant and diverse proteome. Nectar proteins (nectarins) studied thus far either display anti-microbial properties (Carter and Thornburg, 2004; Kram et al., 2008; Hillwig et al., 2010, 2011; Zhou et al., 2016) or modify carbohydrates (González-Teuber et al., 2010; Nepi et al., 2011a, 2012). A nectar redox cycle discovered in *Nicotiana* nectar is based on anti-microbial nectarins that produce hydrogen peroxide, which inhibits microbial infection of the nectary (Carter and Thornburg, 2000, 2004; Carter et al., 2007). On occasion, the microbial defense function and carbohydrate modification reactions overlap. For example, in *Cucurbita pepo* nectar the degradation of pathogen elicitor xylans by β-xylosidases can suppress pathogen infection (Nepi et al., 2011a, 2012).

Cucurbita maxima cv. Big Max is an ideal system to study sex-dependent variations of nectar, because it is a monoecious plant with unisexual flowers. Male flowers of *C. maxima* produce three times less nectar than females and out-number the female flowers 3:1 (Ashworth and Galetto, 2002). In both the male and female flowers, nectariferous tissue lines the adaxial receptacle surface. Secretion of sucrose-dominant nectar produced by starch hydrolysis begins at dawn the day of anthesis and ceases by noon at which point reabsorption of unconsumed nectar occurs (Ashworth and Galetto, 2002). Detailed studies of nectar dynamics in *C. pepo* have found significant sex-dependent variation when comparing the nectar sugar concentration, nectar volume, and rates of nectar production (Nepi et al., 2001).

The main objective of this study was to determine whether sex-dependent variation occurs in nectar composition at the level of both the metabolome and proteome, and secondarily to define potential metabolic links between the proteomes and the production of nectar metabolites. Thus, the combined application of metabolomics and proteomics analyses better define nectar biology of *Cucurbita maxima* cv. Big Max. The nectar of male and female flowers was analyzed using a GC-MS based untargeted metabolomics approach, as well as targeted amino acid profiling. For the first time in cucurbits, the proteomes were examined using LC-MS and iTRAQ (isobaric tag relative and absolute quantitation) to measure nectary protein expression. The collected omics-data were interpreted in the context of two models of nectar secretion, the merocrine and eccrine models (Roy et al., 2017).

MATERIALS AND METHODS

Plant Materials, Growth Conditions, Sample Collection

Seeds of *Cucurbita maxima* cv. Big Max were sown in 4-inch peat pots in a greenhouse. Approximately 2 weeks later, 17 seedlings that were at the two-leaf developmental stage were transplanted to a field plot located at the North Central Regional Plant Introduction Station, Ames, IA, United States (42°00′40.8″N 93°39′46.9″W). Plants were enclosed by a 4.5 m × 12 m × 2 m polyethylene (natural amber) mesh cage to reduce accessibility by insects and the consumption of nectar by pollinators. All nectar and nectary samples were collected at anthesis between 8:00 am and 11:00 am. Flowers were removed from the plant before collecting nectar using an AlphαPette^{TM} pipette with sterile tips. Nectary tissue was then dissected from the flower using a sterile scalpel. Nitrile gloves were worn during all collections. All samples were immediately flash-frozen and stored in liquid nitrogen before long term storage at −80°C.

Nectar Metabolite Extraction and Analysis

Untargeted Metabolomics

An untargeted metabolomics extraction method was adapted from Schmidt et al. (2011). Each extraction used 20 μL of nectar collected from a single flower. For biological replication purposes, extracts were prepared from at least six independent male and female flowers, and they were processed and analyzed individually without pooling. Prior to the extraction, internal standards (5 μg nonadecanoic acid and 2 μg ribitol) were added to the nectar sample. The mixture was immediately incubated for 10 min with 3.5 mL of hot methanol (60°C) followed by sonication for 10 min. Chloroform (3.5 mL) and water (3 mL) were added and the mixture was vortexed after the addition of each solvent. The mixture was centrifuged, and the top polar, and bottom non-polar layers were recovered separately. The entire non-polar layer (3 mL) and 2 mL of the polar layer were transferred to individual 2 mL screw-cap glass vials and dried overnight by lyophilization. The analysis of predominant sugars (glucose, fructose, and sucrose) was conducted with a 1-μL sample of nectar, which was spiked with 25 μg ribitol and the

mixture was dried overnight by lyophilization. The dried polar extracts and the predominant sugar preparations underwent methoximation for 90 min with 20 mg mL^{-1} methoxyamine hydrochloride in pyridine at 30°C with continuous agitation. All samples including the dried non-polar extracts were silylated for 30 min at 60°C with BSTFA/TMCS (*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide/Trimethylchlorosilane).

The predominant sugar samples were diluted with 1 mL pyridine. Samples were analyzed using a GC/GC-MS consisting of an Agilent Technologies Model 6890 gas chromatograph equipped with an Agilent HP-5ms Inert (30 m, 0.25 mm, 0.25 μm) column and a low thermal mass (LTM) oven, which was coupled to Model 5975C mass spectrometer. GC was conducted with a helium gas flow rate of 1 mL min^{-1}, 1 μL injection, and a temperature gradient of 80°–320°C at a rate of 5°C min^{-1}. A heart-cut method, which diverted gas flow to a secondary LTM column at the elution times for fructose, glucose, and sucrose, was utilized to analyze the minor components of the polar extracts. Deconvolution and integration of resulting spectra was performed with AMDIS (Automated Mass Spectral Deconvolution and Identification System) software. Analyte peaks were identified by comparing mass spectra and retention indices to the NIST14 Mass Spectral Library and when possible, to authentic standards to confirm chemical identification (Stein, 1999).

Targeted Amino Acid Analysis

Analysis of amino acids was performed using the Phenomenex EZ:Faast^{TM} kit for free amino acids (Torrance, CA, United States). Each sample (60 μL nectar per extraction) consisted of nectar pooled from four individual flowers. Six replications were analyzed for each sex. Sample preparation from solid phase extraction to derivatization were completed according to the manufacturer with one adjustment: after addition of the norvaline internal standard to each sample, 125 μL of 10% propanol/20 mM HCl was added to acidify the sample. Following derivatization, samples were concentrated under a stream of nitrogen gas before amino acids were analyzed using an Agilent Technologies model 6890 gas chromatograph coupled to a model 5973 mass selective detector capable of electrical ionization (EI). The GC-MS instrument settings followed the manufacturer's recommendations.

Nectar Proteomics

Nectar samples were collected from three individual flowers of both male and female flowers, and these samples were pooled to average biological differences among the two flower types. These pooled nectar samples were analyzed individually for both male and female flowers. Nectar samples were first reduced with dithiothreitol for 30 min at 37°C and alkylated with iodoacetamide for 30 min at 37°C. Each sample was digested with 2 μg trypsin for 16 h at 37°C). Desalting was completed using a Waters HLB Oasis column followed by concentration in a Speed-Vac. Peptide mixtures were rehydrated to 50 μL using a solution of 2% acetonitrile and 2% formic acid. Six microliters were injected for LC-MS/MS analysis using a Thermo Scientific EASY-nLC II system coupled to an LTQ Orbitrap Velos Pro mass spectrometer equipped with a Nanospray Flex source. The LC system utilized a Magic C-18AQ reversed-phase pre-column (100 μm I.D., 2 cm length, 5 μm, 100 Å) and in-house prepared reversed-phase nano-analytical column packed with Magic C-18AQ (75 μm I.D., 15 cm length, 5 μm, 100 Å). The solvent system consisted of buffers A (2% acetonitrile, 0.1% formic acid) and B (90% acetonitrile, 0.1% formic acid) with a 90 min linear gradient (0 min: 5%B; 90 min: 30%B; 2 min: 100%B; 8 min: 100%B) at a flow rate of 300 nL min^{-1}. Orbitrap nano-electrospray ion source was set to a voltage of 2.5 kV and capillary temperature of 250°C. The scan m/z range was 400–2000. The ten most intense ions (charge state 2–4 exceeding 50,000 counts) were selected for ion trap collision induced dissociation (CID) and detection in centroid mode. Common human keratin and porcine trypsin peptide masses were excluded from MSMS selection during the analysis.

Nectary Proteomics

Protein Extraction and iTRAQ Labeling

Each biological replicate consisted of nectary tissue from a single flower with a total of two female replicates and five male replicates. To extract proteins, nectaries were pulverized under liquid nitrogen and solubilized in 4 M urea/0.1 M triethylammonium bicarbonate (TEAB). Proteins were precipitated overnight in acetone and dissolved in 4 M urea/0.1 M TEAB.

Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay. Ten volumes of acetone at −20°C were used to precipitate 100 μg of extracted protein overnight. The resulting protein pellet was dissolved in 0.5 M TEAB/0.2% sodium dodecyl sulfate for 4 h at 4°C before reduction with 50 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP) for 1 h at 60°C. Alkylation with 200 mM methyl methanethiosulfonate (MMTS) at room temperature for 10 min was completed prior to overnight in-solution digestion at 37°C with 10 μg trypsin prepared in 100 mM TEAB. Digests were dried in a Speed-Vac before rehydration with 30 μL of 0.5M TEAB/50 μl isopropanol. iTRAQ labels were added to each sample before being pooled and concentrated to a final volume of approximately 100 μL using a Speed-Vac.

Chromatography and Mass Spectrometry

The iTRAQ labeled peptide sample was fractioned and concatenated using an Agilent 1290 HPLC with a Waters XBridge C18 column (250 mm × 4.6 mm, 5 μm, 300 Å) and solvent system consisting of buffers A (10 mM ammonium hydroxide, pH10) and B (80% acetonitrile, 10 mM ammonium hydroxide, pH 10). The column was equilibrated in buffer A at a flow rate of 0.75 mL min^{-1} before a gradient of 5–45% buffer B was applied over 75 min. Fractions were collected every minute for 96 min, concentrated by lyophilization, and concatenated into 24 fractions by combining every 24th fraction. Fractions were de-salted using C18 StageTips and rehydrated with 20 μL of 2% acetonitrile/3% formic acid. For LC-MS/MS peptide sequencing, 5 μL aliquots of each fraction were injected into a Thermo Scientific EASY-nLC II system coupled to an LTQ Orbitrap Velos Pro mass spectrometer equipped with a Nanospray Flex source.

The same columns, solvent system, and mass spectrometer parameters as described for nectar peptide sequencing in the previous section were used with the following adjustments. Peptides were separated using a 120 min gradient (0 min: 5-% B; 100 min: 40-% B; 5 min: 80-% B; 2 min: 100-% B; 13 min: 100-% B). The scan *m/z* range was set to 400–1800. The top 15 most abundant ions with charge states of 2–4, exceeding 20,000 counts were selected for HCD FT MS/MS fragmentation (FTMSMS scans 2–16) and detection in centroid mode.

Proteomics Data Processing

The nectar and nectary proteome datasets were similarly processed with raw files being created by XCalibur 3.0.63 software and analyzed with Proteome Discoverer (v 1.4.0.228, Thermo Scientific) and were searched against the Uniprot-SwissProt and TrEMBL databases. Nectary dataset search parameters used an MS/MS tolerance of 15 mmu, fixed modification: Methylthio (C), iTRAQ8plex (K), and iTRAQ8plex (N-term), and variable modifications: Oxidation (M), Deamidated (NQ), iTRAQ8plex (Y). The resulting identified proteins underwent statistical validation and filtering using the Scaffold (v 4.6.0 Proteome Software, Inc., Portland, OR, United States) in which the peptide threshold was set to 95% and the minimum number of peptides was set at two. Proteins of non-plant origin were manually removed from datasets. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[1] via PRIDE (Vizcaíno et al., 2016) partner repository, with the dataset identifier PXD009810 and 10.6019/PXD009810.

Statistical Analyses

Relative metabolite concentrations between male and female nectars were compared using a two-tailed independent samples *t*-test with resulting *p*-values corrected for multiple testing using the Benjamini and Hochberg's method. A Mann Whitney test with Benjamini and Hochberg method for multiple testing correction was used to calculate *p*-values based on the log fold change of protein abundance between male and female nectaries. To visualize proteins with significant differences in abundance between male and female nectaries, adjusted *p*-values were negative $\log_{10}$ transformed and plotted against the $\log_2$ fold difference of protein abundance between male and female in a volcano plot.

Gene Ontology (GO) slimming analysis of nectary proteome annotations was completed using GSEABase (Morgan et al., 2017) with annotations mapped up to the generic GO slim set of terms developed by GO Consortium (The Gene Ontology Consortium, 2000, 2017). GO enrichment analysis of the nectary proteome was implemented using topGO: Enrichment Analysis for Gene Ontology (Alexa and Rahnenfuhrer, 2016) with prior protein-to-GO term mapping completed using the UniProt GO annotation database (Barrell et al., 2009). A Fisher's exact test was completed to test for enrichment of GO terms using nectary proteins as the background and differentially expressed proteins as the test group.

[1]http://www.proteomexchange.org/

RESULTS

Nectary Morphology

In both male and female flowers, the nectary tissue lines the adaxial surface of the receptacle. Morphology and nectary environmental exposure varies by sex. Nectariferous tissue encircles the style column forming a trough for the accumulation of the nectar (**Figures 1A,B**). This nectary position leaves female nectar easily accessible to pollinators. The male nectariferous tissue forms a bowl-like structure below the filaments with the nectar only accessible through slits between pairs of fused filaments (**Figures 1D,E**). Nectaries of both sexes heavily stained black with Lugol indicating that the parenchyma tissue is abundant in amylose-rich starch (**Figures 1C,F**).

GC-MS Identification of Nectar Metabolites

Untargeted (GC/GC-MS) and targeted (amino acids) analysis of the nectar metabolome of *C. maxima* led to the detection of 88 analytes, of which 40 could be chemically identified. Classes of identified metabolites from highest to lowest concentrations included sugars, amino acids, sugar alcohols, organic acids, aromatics, esters, and diols. Untargeted metabolite profiling of male and female flowers of *C. maxima* detected a total

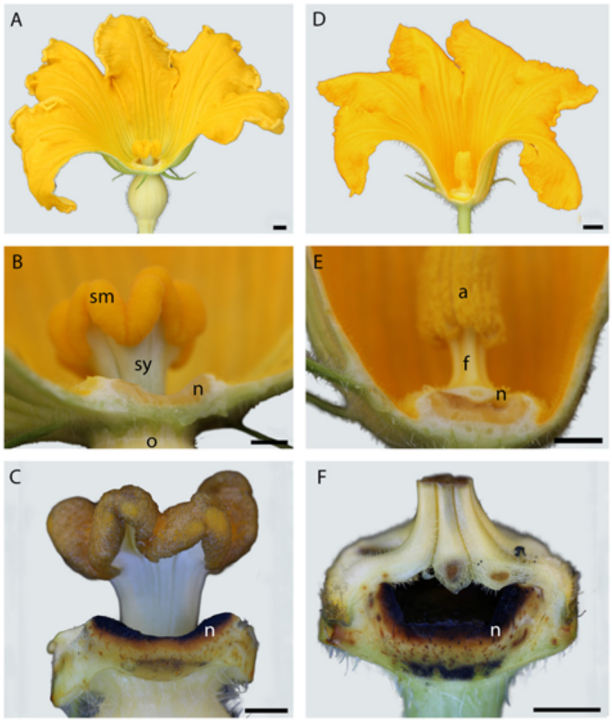

FIGURE 1 | Comparison of female **(A)** and male **(D)** *Cucurbita maxima* flower and nectary morphology. Longitudinal sections of female (left) and male (right) *Cucurbita maxima* flowers. Nectaries of both line the receptacle cavity **(B,E)** and stain black in Lugol potassium iodide solution **(C,F)**. n, nectary; sm, stigma; sy, style; o, ovary; a, anther; f, filament. Scale bars for **A** and **D** = 50 mm; **B, C, E, F** = 5 mm.

of 54 analytes (Supplementary Table 1). Targeted profiling of amino acids detected 34 metabolites with 16 identified as proteinaceous amino acids and three as non-proteinaceous amino acids (**Table 1**). Comparison of the molar percentage of these analytes revealed that male nectar contains significantly more non-essential amino acids, and female nectar has a higher proportion of non-proteinaceous amino acids (**Figure 2**). A total of 29 analytes were found to differ significantly in abundance between male and female nectar (**Figure 3**). Of the 29 analytes, 12 were chemically identified, and whereas glucitol was only detected in male nectar, both glycolic acid and phosphate were exclusively detected in female nectar. Regardless of the flower sex, *C. maxima* nectar was sucrose-dominant with a S/[G + F] ratio above 1 (**Figure 4**). Sucrose concentration was significantly greater in female nectars and contributes to a significantly higher S/[G + F] ratio (p-value = 0.02, **Figure 4**).

TABLE 1 | Amino acids identified in *Cucurbita maxima* nectar reported as mean ± SE (n = 6).

Amino Acid	Concentration (μM)		% of total amino acid	
	Female	Male	Female	Male
*Alanine	117 ± 14	212 ± 40	42.8 ± 3.4	52.9 ± 3.4
*Glycine	3.6 ± 0.7	7.6 ± 1.3	1.3 ± 0.2	1.9 ± 0.2
Serine	5.7 ± 1.4	9.5 ± 2.2	1.9 ± 0.4	2.4 ± 0.5
Proline	30.5 ± 4.6	45.2 ± 9.2	11.3 ± 1.6	12.6 ± 3.2
Asparagine	10.2 ± 1.8	7.7 ± 1.6	3.6 ± 0.4	2.1 ± 0.6
Aspartic acid	6.9 ± 2.8	5.2 ± 0.7	2.4 ± 0.8	1.4 ± 0.2
Glutamic acid	11.9 ± 2.4	12.5 ± 1.6	4.4 ± 0.9	3.3 ± 0.5
Tyrosine	0.52 ± 0.15	0.75 ± 0.17	0.17 ± 0.04	0.19 ± 0.04
*Tryptophan	0.23 ± 0.07	0.57 ± 0.11	0.08 ± 0.02	0.16 ± 0.03
Valine	11.9 ± 1.7	14.4 ± 2.5	4.3 ± 0.4	3.6 ± 03
Leucine	3.4 ± 0.4	4.8 ± 1.4	1.2 ± 0.1	1.1 ± 0.2
Isoleucine	11.9 ± 2.0	11.8 ± 2.8	4.4 ± 0.7	2.9 ± 0.6
Threonine	1.3 ± 0.4	2.2 ± 0.3	0.48 ± 0.11	0.59 ± 0.09
Methionine	1.4 ± 0.3	1.9 ± 0.6	0.51 ± 0.10	0.45 ± 0.09
Phenylalanine	11.5 ± 1.8	14.0 ± 1.7	4.2 ± 0.6	3.6 ± 0.3
Lysine	0.24 ± 0.09	0.72 ± 0.31	0.09 ± 0.03	0.19 ± 0.08
β-Alanine	13.1 ± 2.7	15.5 ± 3.1	4.6 ± 0.7	4.4 ± 1.1
GABA	32.9 ± 4.3	21.8 ± 4.4	12.1 ± 1.3	5.9 ± 1.4
4-Hydroxyproline	0.87 ± 0.66	0.54 ± 0.12	0.33 ± 0.24	0.14 ± 0.03

*GABA, γ-aminobutyric acid. *Indicates metabolites with significantly different concentration between male and female nectar, p-value <0.05.*

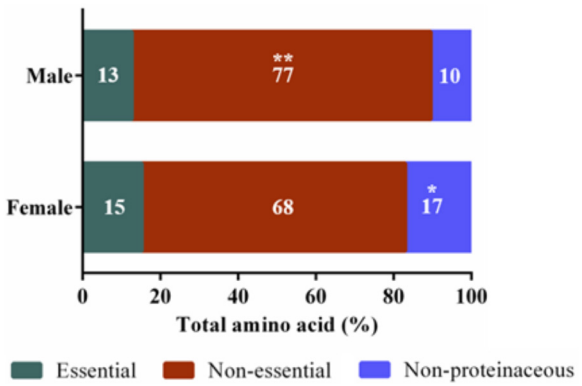

FIGURE 2 | Amino acid categories of *Cucurbita maxima* male and female nectars. Essential amino acids included tryptophan, valine, leucine, isoleucine, threonine, methionine, phenylalanine, and lysine **p-value 0.004, *p-value 0.03. n = 6, with each replicate consisting of nectar pooled from four flowers.

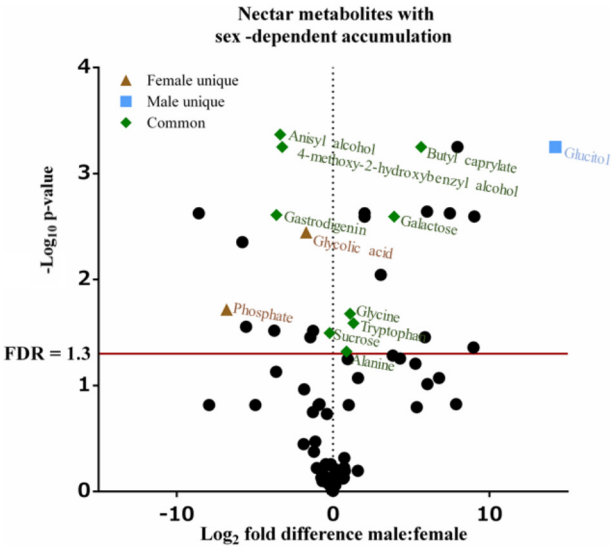

FIGURE 3 | Volcano plot of *Cucurbita maxima* nectar metabolome. Points above the red FDR line represent metabolites with p-values <0.05. n = 6, with each replicate consisting of nectar from single flowers.

Nectar Proteome

The pooled nectar proteome combined from three individual male and female flowers consists of 45 detected proteins (Supplementary Table 2), 33 of which are present in nectar from both sexes. Two proteins are unique to female nectar and 10 are unique to male nectar. Unique female nectar proteins include galactinol-sucrose galactosyltransferase 2 and cysteine proteinase inhibitor. In the male nectar, eight of the ten unique proteins were characterized as 4-alpha-glucanotransferase, aconitate hydratase, enolase 1, fructose-bisphosphate aldolase, invertase, polygalacturonase, and two different 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferases. Two unique proteins were uncharacterized proteins from the Uniprot Trembl database. More rigorous sampling in future proteomics analyses may further expand upon these findings, representing the first effort toward cataloging the nectarins of *C. maxima* male and female nectar.

Nectary Proteome

A total of 339 proteins were detected in the nectaries of male and female *C. maxima* flowers using iTRAQ (Supplementary Table 3). To gain a broad overview of functional classifications for the nectary proteome, GO slim analysis was implemented.

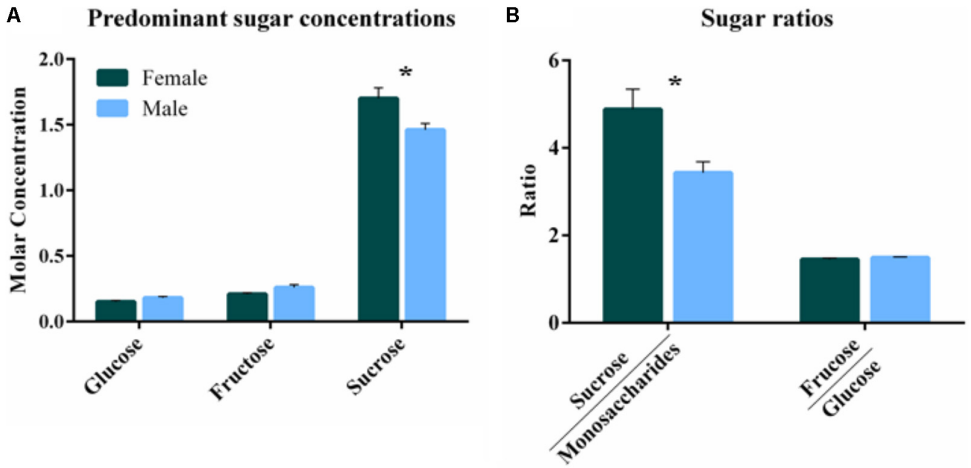

FIGURE 4 | Comparison of *Cucurbita maxima* predominant sugars by flower sex. **(A)** Mean molar concentration ± SE of the predominant sugars. **(B)** Ratios of the disaccharide (sucrose) to the monosaccharides (glucose and fructose) and fructose to glucose for each flower sex. *p-value <0.05. n = 6, with each replicate consisting of nectar from single flowers.

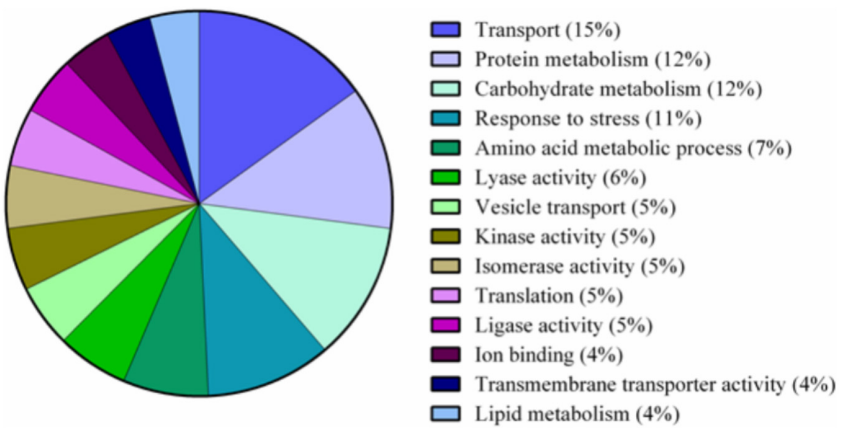

FIGURE 5 | Pie chart of functional classification of proteins found in the nectaries of *Cucurbita maxima*. GO slim categories from the Gene Ontology Consortium were used. Percentages following category name represent the percentage of annotations falling within that category from the top 48% of all GO annotations.

This revealed a high abundance of proteins related to transport, protein metabolism, carbohydrate metabolism, response to stress, and amino acid metabolic process (**Figure 5**). Statistical comparisons of relative protein abundance revealed that 45 proteins displayed differential expression between male and female nectaries (p-value <0.05); 20 of these proteins were more abundant in male nectaries and 25 were more abundant in female nectaries. All 45 proteins have at minimum GO annotation inferred by homology, and descriptive identities are available for 38 of these significant proteins (**Figure 6**). GO enrichment analysis was completed separately for male and female abundant proteins at the three categories of ontology: biological process, molecular function, and cellular component. The most detailed enriched child GO terms for biological process and molecular function are displayed in **Figure 7**. Two cellular component terms, cytosol and cytoplasmic, are female nectary-enriched, while no term is male nectary-enriched. Complete lists of input GO IDs and enriched terms are listed in Supplementary Tables 4 and 5, respectively. Female nectary-enriched GO terms relate to transmembrane transport of ions, magnesium ion binding, response to water deprivation, and carboxy-lyase catalytic activity. Most male nectary-enriched GO terms are related to phenylalanine ammonia-lyase, an enzyme involved in phenylpropanoid biosynthesis (**Figure 7**). Additional enriched GO terms include cellular oxidant detoxification, negative regulation of cellular process, response to heat, and membrane organization.

DISCUSSION

The synthesis and secretion of nectar is a highly dynamic process, which is only recently beginning to be understood through the robustness of "omics" technologies. Presently, there

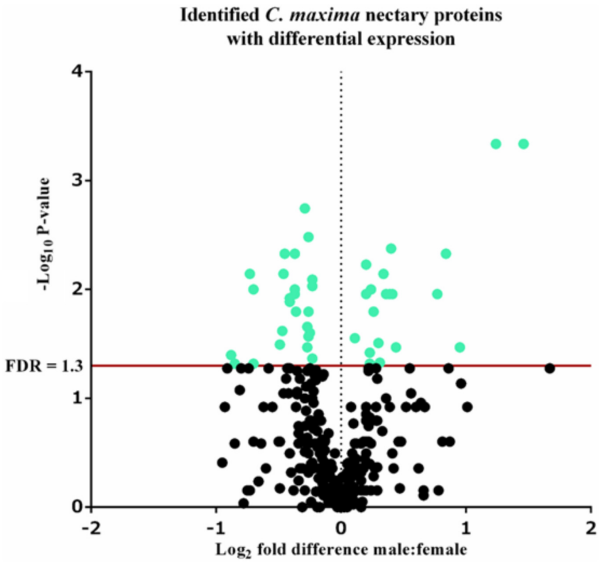

FIGURE 6 | Volcano plot of *Cucurbita maxima* nectary proteome determined by iTRAQ using two female and five male biological replicates with each replicate consisting of the nectary tissue from a single flower. Green points above the red FDR line represent proteins with adjusted *p*-values <0.05.

are two competing models of nectar secretion supported by ultrastructural analyses or molecular genetic studies. In the first model, merocrine (granulocrine), pre-nectar metabolites are transported symplastically through plasmodesmata until they reach cells near the nectary surface, where they are packed into ER or Golgi body vesicles for later fusion with the plasma membrane and secretion. The second model, eccrine, depends on plasma membrane localized pores and transporters instead of vesicles for exporting nectar metabolites from the nectary cells (Roy et al., 2017). This model is supported by the conservation of SWEET9, a plasma membrane sucrose uniporter, within mature nectaries of Brassicaceae and Solanaceae (Lin et al., 2014). Once nectar is secreted, it is far from a complex static solution of primarily sugars. Rather, nectar is in a dynamic equilibrium, responsive to environmental conditions and can undergo post-secretory modifications via the action of catalytic nectarins which act on carbohydrates or generate anti-microbial agents such as hydrogen peroxide (Carter and Thornburg, 2004; González-Teuber et al., 2010; Nepi et al., 2011a,b). The primary objective of the current study was to examine potential sex-dependent variation in *C. maxima* nectar composition at the level of the metabolome and proteome extending existing knowledge of biologically relevant sex-dependent nectar variation with regards to nectar composition and rates of nectar production (Nepi et al., 2001; Ashworth and Galetto, 2002). Secondarily, this study aimed to propose metabolic links between nectar metabolites and proteins present in the nectary and nectar proteomes.

Nectar Metabolomics

Compared to the nectar of male flowers, female nectar of *C. maxima* has significantly more sucrose and a higher sucrose

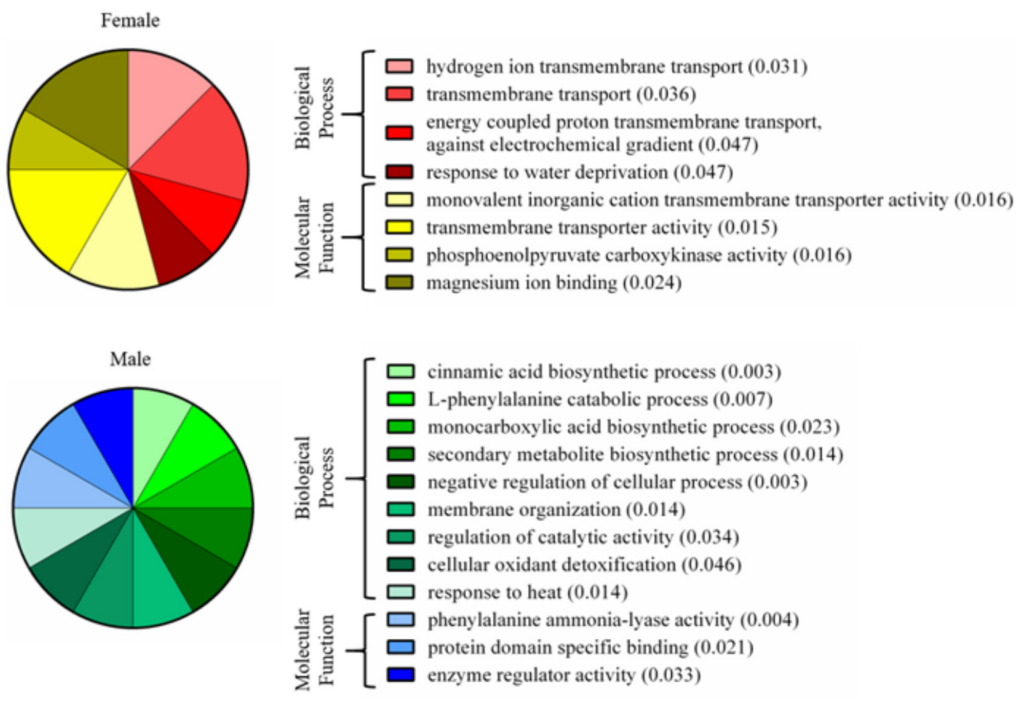

FIGURE 7 | Enriched gene ontology terms of nectary proteins that are differentially expressed between male and female flowers. Pie charts display the most specific enriched GO terms associated with proteins of increased abundance in female or male nectaries. Numbers in parentheses are *p*-values calculated from a Fisher's exact test for enrichment.

to hexose ratio. These findings contrast with previous studies of *C. pepo* and *C. maxima* (Nepi et al., 2001; Ashworth and Galetto, 2002) that found little difference in abundances of the three predominate sugars between male and female nectars. This variation in the findings between the studies may be due to differences in environmental growing conditions of the plants as well as variation in species and cultivar. This may be particularly significant in light of the fact that these sugars influence defining characteristics of nectar, such as viscosity and its ability to attract pollinators (Baker and Baker, 1983). A second sugar, galactose, present at much lower concentrations than sucrose, glucose, and fructose, was significantly less abundant in female nectar. Because bees can easily judge sugar composition and nectar volume (Hendriksma et al., 2014), the variation in both sucrose and galactose content observed in *C. maxima* nectars may influence the degree to which bees are more attracted to female flowers (Ashworth and Galetto, 2002).

Amino acids are the second most common class of metabolites that occur in nectar, but their concentrations are 100 to 1,000 times less than the predominant sugars (Roy et al., 2017). In the present study, 16 proteinaceous amino acids and three non-proteinaceous amino acids were identified in both male and female nectar of *C. maxima*. Over 70% by mole of the identified amino acids were accounted by alanine, proline, GABA, and β-alanine. Although this is similar to the nectar of *C. pepo* (Nepi et al., 2012), there is a striking difference in the relative proportion of proline and alanine; in *C. pepo* proline is the most abundant amino acid followed by alanine (30% and 5% respectively) (Nepi et al., 2012), in *C. maxima* nectar, their relative order is reversed, with alanine being the most abundant amino acid (40%), followed by proline (11%). Proline often occurs as an abundant nectar amino acid, and has multiple effects on bees, including providing a desirable flavor and serving as a muscle stimulant giving a quick burst of energy for flight take-off (Carter et al., 2006; Teulier et al., 2016). The finding of two relatively high abundant non-proteinaceous amino acids, GABA and β-alanine, in *C. maxima* nectars agrees with commonly observed amino acid profiles of floral nectars (Nepi et al., 2012). They are both thought to promote insect flight, while GABA is also implicated as an antimicrobial agent used by plants in response to wounding (Chevrot et al., 2006). Since GABA is also a neurotransmitter (Nepi, 2014), it is possible that it may directly influence bee behavior.

The most significant differences between male and female nectars, in regard to amino acids, was the relative abundance of tryptophan, alanine, and glycine, which were specifically more concentrated in male nectar. These amino acids appear to alter bee feeding preferences, with tryptophan and alanine functioning as bee attractants, while glycine is a deterrent (Bertazzini et al., 2010; Hendriksma et al., 2014). Based on these previous studies, it is unclear whether the statistically significant variation in tryptophan, alanine, and glycine would influence bee feeding preferences between male and female flowers. Studies are needed to determine the biologically relevant ratio of the attractants (alanine and tryptophan) to deterrents (glycine) needed to alter bee preferences as mixtures of amino acids can have synergistic effects on bee preferences. When the proportions of essential, non-essential, and non-proteinaceous amino acids are compared by sex, we found that the male nectar has a significantly higher proportion of non-essential amino acids, largely due to increased concentrations of alanine and glycine. Female nectar contained more non-proteinaceous amino acids, specifically GABA (p-value = 0.009) which as previously stated may confer anti-microbial properties important in keeping the gynoecium free of pathogenic infection.

In addition to sugars and amino acids, nectar often contains a diversity of primary and secondary metabolites whose functions are wide ranging and include pollinator rewards, preservatives, and defense against pathogens (Stevenson et al., 2017). In our study, additional primary metabolites (glucitol, glycolic acid, and phosphate) and secondary metabolites (4-methoxy-2-hydroxybenzyl alcohol, anisyl alcohol, butyl caprylate, and gastrodigenin) displayed sex-dependent difference in accumulation. To our knowledge, no nectar-specific functions are reported for these metabolites, although the sex-dependent accumulation of these metabolites may indicate that they influence pollinator attraction to male and female flowers. Specifically, glucitol was only detected in male nectar, whereas glycolic acid and phosphate were restricted to female nectar. Butyl caprylate, a fragrant ester, which was more abundant in male nectar, has previously been detected in floral volatile profiles of orchids (Kaiser, 1993). In female *C. maxima* nectar, 4-methoxy-2-hydroxybenzyl alcohol, anisyl alcohol, and gastrodigenin are present at higher concentrations as compared to male nectars. Anisyl alcohol, similar to butyl caprylate, is not only a floral scent present in orchids (Kaiser, 1993) but also occurs in anise, honey, and vanilla (Scognamiglio et al., 2012). Gastrodigenin, also known as 4-hydroxybenzyl alcohol, is a known antioxidant occurring in a variety of plants (Lim et al., 2007).

Nectar Proteome

Prior characterization of nectarins have indicated that these proteins function as either anti-microbials or as enzymes that alter nectar carbohydrate chemistries. Consistent with the latter observation, 9 of the 10 proteins that are unique to male nectar are enzymes that act on carbohydrates, the exceptions being 5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase. These carbohydrate-modifying enzymes include invertase, which catalyzes the hydrolysis of sucrose to glucose and fructose. Invertases have previously been reported in other nectars and studied extensively in *Acacia* extrafloral nectar and *C. pepo* floral nectar (Heil et al., 2005; Nepi et al., 2012). Six of the characterized male unique proteins (4-alpha-glucanotransferase, 5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase, aconitate hydratase, enolase 1, fructose-bisphosphate aldolase, and polygalacturonase) have not previously been reported in nectar, but annotation data indicate that they are either located in cytoplasm of cells or extracellular space, supporting their detection in *C. maxima* nectar.

Female nectar contains two unique nectarins, a cysteine proteinase inhibitor and galactinol–sucrose galactosyltransferase 2. The first of these has previously been reported in the

floral nectar proteome of *Liriodendron tulipifera* (Zhou et al., 2016), but the latter has not been reported in nectars. The galactosyltransferase has the potential to modify the carbohydrate profile of female nectar as it functions in galactose metabolism, generating myo-inositol and raffinose from galactinol and sucrose.

In addition to the sex-specific nectarins, 33 other proteins were detected in the nectar proteome of both *C. maxima* flower sexes. Several of these were previously reported in nectars of other species, including malate dehydrogenase in petunia nectar (Hillwig et al., 2011), β-glucosidase in nectar of *Acacia hindsii* and *A. collinsii* EFN (González-Teuber et al., 2010), α-galactosidase in common tobacco nectar (Zha et al., 2012), and glutathione *S*-transferase and a heat shock protein both of which occur in the nectar of *Liriodendron tulipifera* (Zhou et al., 2016). A second group of nectarins (i.e., adenosylhomocysteinase 1, β-galactosidase, and α-glucan phosphorylase) were identified in both male and female *C. maxima* nectars, but they had not previously been reported in nectars of other species. These proteins were also undetectable in the nectary proteome of *C. maxima* flowers. The absence of these proteins in the proteome of the nectary, where they are synthesized, may indicate that these proteins are efficiently and rapidly secreted into the nectar. It is also possible that the complexity of the nectary proteome masks the identification of nectar proteins at their site of synthesis.

Nectary Proteome

The major functional classifications of the *C. maxima* nectary proteome includes proteins involved in transport, protein metabolism, carbohydrate metabolism, response to stress, and amino acid metabolism (**Figure 4**), and these are similar to those found in *Acacia cornigera* (Orona-Tamayo et al., 2013) and *Ricinus communis* (Shah et al., 2016) extrafloral nectary proteomes. These functional classifications are expected as carbohydrates and amino acids are the most abundant nectar metabolites and require extensive transport within the nectary. GO enrichment analysis of nectary proteins with increased female abundances indicate that female-enriched GO terms are associated with proteins functioning as plasma membrane proton pumps and central metabolism, specifically gluconeogenesis, glycolysis, lipid metabolism, and the citric acid cycle. Proteins associated with male nectary-enriched GO terms were related to cinnamic acid biosynthesis and neutralization of superoxide radicals and hydrogen peroxide. If pumpkin nectaries generate high levels of reactive oxygen species (ROS), like tobacco (Carter and Thornburg, 2004; Carter et al., 2007), it would not be surprising if they also contain mechanisms to mitigate their potentially damaging reagent.

As a whole, the nectary proteome in conjunction with previous cucurbit nectary literature supports an eccrine model of nectar secretion where plasma membrane (PM) H-$^+$-ATPase provides the energy for active transport of solutes into the apoplasm of *C. maxima* nectaries. In the current study, functional classification of nectary proteins and GO term enrichment analyses both revealed an abundance of ATPase transmembrane transporters specific for hydrogen ions, indicating the important role of PM-H-$^+$-ATPase in active *C. maxima* nectaries. This finding agrees with the pressure-driven mass flow model of nectar movement from parenchyma tissue into the apoplast, in which PM-H-$^+$-ATPase provides energy for active transport of solutes into the apoplast creating an osmotic gradient for the movement of water through aquaporins. The resulting hydrostatic pressure in the apoplast produces mass flow of nectar out of the nectary tissue and to the surface (Vassilyev, 2010). Additionally, it has also been suggested that nectar secretion in *Cucumis sativus* requires PM-H-$^+$-ATPase, as ATPase-specific activity peaks at anthesis (Peng et al., 2004).

Previous ultrastructural analyses of *C. pepo* demonstrate that the nectary cells are devoid of extensive ER and Golgi making the vesicle dependent merocrine model unfavorable when compared to the eccrine model (Nepi et al., 1996). While the eccrine model may predominate, merocrine is still needed for vesicular-based transport of nectarins, and may be important in *C. maxima* nectaries as vesicle transport is frequency functional classification of its proteome (**Figure 4**) (Roy et al., 2017). The eccrine model of nectar synthesis and secretion that is supported by molecular evidence from Brassicaceae and Solanaceae expresses four metabolic processes: (1) starch degradation, (2) sucrose synthesis, (3) export of sucrose into apoplasm via SWEET9, and (4) extracellular hydrolysis of sucrose via CELL WALL INVERTASE4 (CWINV4) (Ruhlmann et al., 2010; Lin et al., 2014; Thomas et al., 2017). The *C. maxima* nectary proteome determined herein supports the occurrence of the first two of these processes, as both a β-amylase for starch hydrolysis and sucrose-phosphate synthase that function in sucrose biosynthesis are present. Homologs of SWEET9 and CWINV4 were not identified within the nectary proteome under the specified data filtering conditions. Moreover, as a transmembrane protein, SWEET9 may not have been extracted from the nectary tissue as the methodology was not ideal for extraction of membrane proteins. CWINV4 may not be highly expressed in *C. maxima* nectaries which produce a sucrose dominant nectar as compared to the hexose dominant nectar produced by the Arabidopsis nectaries; the expression of CWINV4 is essential for functional development of nectaries in Arabidopsis (Ruhlmann et al., 2010).

Metabolic Links Between Nectar Metabolites and Proteomes

Nectarins commonly alter nectar carbohydrates. In our datasets, significant differences in carbohydrate abundance, specifically galactose and sucrose, may be explained by the unique presence of galactinol–sucrose galactosyltransferase 2 and invertase in the nectar of female and male flowers respectively. Galactose is significantly less in female nectar which also contains galactinol–sucrose galactosyltransferase 2 which is not found in male nectar. This enzyme utilizes galactose as a substrate, leading to the production of myo-inositol and raffinose, a primary transport sugar in cucurbits (Zhang et al., 2010); this may explain why galactose levels are lower in female nectar as compared to male. A second potential example of post-secretory carbohydrate alterations is suggested by the slight but statistically significant reduction in sucrose content of male nectar which contains

an invertase that is not detectable in female nectar. Invertases catalyze the hydrolysis of sucrose to glucose and fructose. The difference in sucrose concentration between male and female nectar may only be slight due to the ability of the male nectary to maintain a nectar equilibrium. In *C. pepo* for example, male flowers can regulate water and sugar content to maintain nectar homoeostasis during secretion (Nepi et al., 2011b). This ability to regulate sugar content may nullify the impact of invertase within the male nectar of *C. maxima*.

CONCLUSION

In this study, we demonstrated an existence of sex-dependent variation in male and female floral nectaries and nectar of *C. maxima* as determined by proteomics and metabolomics. Nectar metabolites that varied in composition range from carbohydrates, amino acids, and specialized metabolites, and the nectarin profiles. Nectarins specific to a single nectar sex were linked to observed differences in the nectar metabolomes. Additionally, the nectary proteome supported aspects of the eccrine model of nectar secretion and pressure-driven mass flow utilizing PM-H-$^{+}$-ATPase.

AUTHOR CONTRIBUTIONS

CJC conceived and planned the research. EC completed sample collection and metabolomics analyses. Proteomics experimental work and analyses were completed by ME, DS, EC, and PvA. Manuscript was written by EC, PvA, and BN.

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Characterization of a L-Gulono-1,4-Lactone Oxidase Like Protein in the Floral Nectar of *Mucuna sempervirens*, Fabaceae

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Floral nectar plays important roles in the interaction between animal-pollinated plants and pollinators. Its components include water, sugars, amino acids, vitamins, and proteins. Growing empirical evidence shows that most of the proteins secreted in nectar (nectarines) are enzymes that can tailor nectar chemistry for their animal mutualists or reduce the growth of microorganisms in nectar. However, to date, the function of many nectarines remains unknown, and very few plant species have had their nectar proteome thoroughly investigated. *Mucuna sempervirens* (Fabaceae) is a perennial woody vine native to China. Nectarines from this species were separated using two-dimensional gel electrophoresis, and analyzed using mass spectrometry. A L-gulonolactone oxidase like protein (MsGulLO) was detected, and the full length cDNA was cloned: it codes for a protein of 573 amino acids with a predicted signal peptide. MsGulLO has high similarity to L-gulonolactone oxidase 5 (AtGulLO5) in *Arabidopsis thaliana,* which was suggested to be involved in the pathway of ascorbate biosynthesis; however, both MsGulLO and AtGulLO5 are divergent from animal L-gulonolactone oxidases. *MsGulLO* was expressed mainly in flowers, and especially in nectary before blooming. However, cloning and gene expression analysis showed that L-galactonolactone dehydrogenase (MsGLDH), a vital enzyme in plant ascorbate biosynthesis, was expressed in all of flowers, roots, stems, and especially leaves. MsGulLO was purified to near homogeneity from raw MS nectar by gel filtration chromatography. The enzyme was determined to be a neutral monomeric protein with an apparent molecular mass of 70 kDa. MsGulLO is not a flavin-containing protein, and has neither L-galactonolactone dehydrogenase activity, nor the L-gulonolactone activity that is usual in animal GulLOs. However, it has weak oxidase activity with the following substrates: L-gulono-1,4-lactone, L -galactono-1,4-lactone, D-gluconic acid-δ-lactone, glucose, and fructose. MsGulLO is suggested to function in hydrogen peroxide generation in nectar but not in plant ascorbate biosynthesis.

Keywords: L-ascorbate biosynthesis, Floral nectar, L-gulonolactone oxidase like protein, L-galactonolactone dehydrogenase, *Mucuna sempervirens* Hemsl, Nectarin

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Abbreviations: ALO, D-arabinono-1,4-lactone oxidase; AsA, L-ascorbic acid; D-GluL, D-gluconic acid-δ-lactone; FAD, flavin adenine dinucleotide; GalLO, L-galactono-1,4-lactone oxidase; GlcUR, D-glucuronate reductase; GLDH, L-galactono-1,4-lactone dehydrogenase; GulLDH, L-gulono-1,4-lactone dehydrogenase; GulLO, L-gulono-1,4-lactone oxidase; GUO, D-gluconolactone oxidase; L-GalL, L-galactono-1,4-lactone; L-GulL, L-gulono-1,4-lactone; SEC, size exclusion chromatography.

INTRODUCTION

L-Ascorbic acid (ascorbate, AsA), is a naturally occurring organic compound belonging to the family of monosaccharides. This compound has antioxidant properties, which help protect against reactive oxygen species (ROS) derived from metabolic activity. AsA also plays an essential role in eukaryotes as an enzyme co-factor in hydroxylation reactions, contributing to diverse processes such as the synthesis of collagen and the demethylation of histones and nucleic acids (Mandl et al., 2009).

L-Ascorbic acid, also known as vitamin C, has multiple applications as a therapeutic for human health, for example in the treatment of common cold, wound healing, and cancer; it is also the vitamin that prevents scurvy. In animals, AsA is synthesized from glucose through intermediates D-glucuronate and L-gulono-1,4-lactone; this is termed the animal pathway. Humans, non-human primates, guinea pigs, bats, and some birds cannot synthesize AsA because L-gulono-1,4-lactone oxidase (GulLO), the terminal enzyme in the biosynthesis process, does not function due to mutation (Chatterjee, 1973). Therefore, these animals including humans need to acquire this vitamin from fresh fruits and green vegetables. Plant-derived AsA is the major source of AsA in the human diet.

L-Ascorbic acid is the most abundant and best characterized water-soluble antioxidant in plants (Foyer and Shigeoka, 2011). Within a plant, AsA mostly accumulates in in photosynthetic organs. The concentration of AsA in cells in green tissues can be up to 5 mM, representing 10% of the total soluble carbohydrate pool (Smirnoff and Wheeler, 2000). As a critical metabolite in plants, AsA has several essential functions in plant physiology, participates in the detoxification of ROS, and has an important role in promoting resistance to senescence and numerous environmental stresses, such as high temperature, dehydration stress, high light, ozone, UV-B radiation, and salt stress. Also, AsA operates as a cofactor, taking part in the regulation of some fundamental cellular processes (e.g., photoprotection, the cell cycle, and cell expansion) and biosynthesis of important plant hormones (e.g., including abscisic acid, jasmonic acid, ethylene, and gibberellic acid) (Smirnoff, 2011; Liang et al., 2017).

The biosynthetic pathways of AsA differ between plants and animal. Plants appear to have multiple pathways for AsA biosynthesis. The primary and most elucidated pathway is the "Wheeler–Smirnoff pathway" which is also called as "D-mannose/L-galactose pathway" or "plant pathway" and start AsA biosynthesis from glucose or mannose (Wheeler et al., 1998, 2015). All genes in this pathway have been identified and at the last step of this pathway, AsA is formed from L-galactono-1,4-lactone in an enzymatic reaction catalyzed by L-galactono-1,4-lactone dehydrogenase (GLDH). Alternative AsA biosynthetic pathways appear to exist in plants, involving galacturonate and glucuronate, but not all enzymes of these pathways have been identified, and little is yet known about their regulation (Bulley and Laing, 2016).

The plant AsA biosynthetic pathway employs GLDH as the terminal enzyme, whereas GulLO has this role in animals. GulLO is deemed to be absent from most of Archaeplastida genomes including higher plants (Wheeler et al., 2015). It is interesting that overexpression of rat GulLO caused an increase in AsA content in tobacco (Jain and Nessler, 2000), potato (Hemavathi et al., 2010), tomato (Lim et al., 2012) and *Arabidopsis* (Lisko et al., 2013). If fed L-gulono-1,4-lactone (L-GulL), detached bean (*Phaseolus vulgaris*) and strawberry (*Fragaria x ananassa*) fruits could convert it to AsA (Baig et al., 1970). GulLO enzyme activity has been detected in hypocotyl homogenates of kidney beans (Siendones et al., 1999), cytosolic and mitochondrial fractions of *Arabidopsis* cell cultures (Davey et al., 1999), and potato tubers (Wolucka and Van Montagu, 2003). An enzyme family exhibiting some similarity to animal GulLO has also been reported in *Arabidopsis* (Maruta et al., 2010). Three putative *Arabidopsis* GulLOs (AtGulLO2, 3, and 5) over-expressed in tobacco BY-2 cell cultures increased AsA after feeding with L-GulL (Maruta et al., 2010). Aboobucker et al. (2017) purified a recombinant *Arabidopsis* GulLO enzyme (AtGulLO5) in a transient expression system. They proved that AtGulLO5 is an exclusive dehydrogenase with an absolute specificity for L-GulL as substrate, thus differing from both existing plant GLDHs and mammalian GulLOs. However, the catalytic efficiency of AtGulLO5 was low.

These findings suggested that there might be an animal AsA biosynthetic pathway analog existing in plants with GulLO as the terminal enzyme. However, up to date, no spontaneous GulLOs had been isolated from plants and no activity from such plant GulLOs has been analyzed. In this study, for the first time, we identified an AtGulLO homolog (named as MsGulLO) in the nectar from *Mucuna sempervirens* Hemsl (Fabaceae), a perennial woody climber bean species that is widely distributed in subtropical regions of China, Bhutan, North East India (West Bengal, Manipur, Sikkim), Japan and Myanmar. We then cloned the full-length cDNA sequence of *MsGulLO* and a L-galactono-1,4-lactone dehydrogenase gene (named as *MsGLDH*) which was used as a reference in the gene expression and phylogenetic analysis. The functional difference between MsGulLO and MsGLDH will be discussed in the context of their phylogenetic relationship and gene expression. MsGulLO was purified to near homogeneity from the nectar using size-exclusion chromatography and the enzymatic activity was assayed *in vitro*. The possible role of MsGulLO in the nectar is also discussed.

MATERIALS AND METHODS

Mucuna sempervirens Floral Nectar Collection, pH, Hydrogen Peroxide, AsA, Glutathione, Sugars, and Protein Content Determination

Three *M. sempervirens* (MS) plants grown in greenhouse at Huangshan University (Anhui province, China) were used in this study. Raw nectar was collected from MS flowers in April 2017. Pooled nectar was filtered through 0.22 μm syringe filters (Millipore) to remove dirt and pollen granules from the samples, and stored at −80°C prior to use. The pH of individual nectar samples from 15 flowers was measured by using

a pH meter (Model FiveGo F2, Mettler Toledo) with an InLab Micro Probe (Mettler Toledo). Total sugar concentration of MS nectar samples was estimated as the Brix value, obtained with a low-volume hand-held refractometer (Eclipse, Bellingham and Stanley, Tunbridge Wells, United Kingdom). The determination of sugars and L-gulono-1,4-lactone in MS nectar was performed with an EClassical 3100 high-performance liquid chromatograph (Elite, Dalian, China) equipped with a refractive index detector (RI-201H, Shodex, Japan). The separation was performed using a carbohydrate column (SC1011, Shodex, Japan), and purified water was used as an eluent for analysis at a flow rate of 0.8 ml min^{-1} at 85°C. The concentration of hydrogen peroxide in MS nectar samples was measured using a commercially available colorimetric assay kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The concentration of total and reduced AsA in nectar was determined following the method of Kampfenkel et al. (1995). Glutathione (GSH) and oxidized GSH (GSSG) in nectar were measured with a GSH/GSSG Assay Kit (Beyotime Biotech Co., Ltd., Shaghai, China). Protein content in the nectar samples was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

Two-Dimensional Gel Electrophoresis (2-DE) and Mass Spectrometry

Because of high protein concentration in MS nectar, filtered nectar was directly used for 2-DE without further concentration. Isoelectrofocusing (IEF) was carried out using a PROTEAN i12 IEF system (Bio-Rad) and a 7-cm Immobiline Dry Strips (linear pH 3–10, Bio-Rad) as described in Ma et al. (2017). Second-dimension electrophoresis was carried out on 12% polyacrylamide gels in a Mini-PROTEAN Tetra system (Bio-Rad) following the manufacturer's instructions. The 2-DE gels were double stained with Coomassie Brilliant Blue G250 and silver nitrate. Samples were run in triplicate.

For protein identification, spots of interest were manually excised from 2-DE gels and subjected to in-gel digestion using trypsin as the protease, followed by protein identification using a 5800 tandem matrix-assisted laser-desorption ionization time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, United States). The combined mass spectrometry (MS) and tandem MS (MS/MS) peak lists were analyzed using GPS (Global Proteome Server) Explorer Software 3.6 (Applied Biosystems) with a Mascot search engine (MASCOT version 2.3; Matrix Science, London, United Kingdom), and searched against the National Center for Biotechnology Information database (NCBIprot 20170707). The taxonomic restrictions were set to NCBI-Other green plants. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the data set identifier PXD010067.

Cloning of *MsGulLO* and *MsGLDH*

For RACE, total RNA samples were isolated from the stylopodium (which contains the nectary) from MS flowers (stage S4 as shown in **Figure 1**) using a RNeasy Plant Mini kit (Qiagen) with an on-column DNase treatment according to the manufacturer's protocol. The quality and concentration of extracted RNA was assessed using a Nanodrop Spectrophotometer (ND-2000, Thermo Fisher Scientific).

A protein detected by the above analysis was determined to be an L-gulonolactone oxidase, here named as MsGulLO. To clone the full-length cDNA of *MsGulLO*, a combination of 3′ and 5′ RACE PCR was performed using a SMARTer RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. A primer named as GulLO-F for 3′ RACE was designed according to the well conserved regions in sequence alignments of five GulLO genes from *Cajanus cajan* (accession no. XM020368474), *Glycine max* (accession no. XM003548358), *Lupinus angustifolius* (accession no. XM019600852), *Medicago truncatula* (accession no. XM013589106), and *P. vulgaris* (accession no. XM007135246).

To clone the reference MS *GLDH* cDNA (here named as *MsGLDH*), a primer named as GLDH-F was designed for 3′ RACE according to the conserved motif sequences of five GLDH genes from *Glycine max* (accession no. NM001249443), *Lupinus angustifolius* (accession no. XM019572875), *Medicago truncatula* (accession no. XM003590185), *P. vulgaris* (accession no. XM007145487), and *Vigna angularis* (accession no. XM017559956). The primers for 5′ RACE were then designed according to the sequence data from 3′ RACE for both *GULO* and *GLDH*. Products of RACE reactions were directly sequenced without any cloning steps. The resulting sequence reads were assembled to generate the full length cDNA sequences of *MsGulLO* and *MsGLDH*. Primer sequences used are detailed in **Supplementary Table S1**. The full length sequences of *MsGulLO* and *MsGLDH* were deposited in GenBank.

Analysis of Gene Expression

To investigate the relative expression of *MsGulLO* and *MsGLDH* transcripts in different plant organs, total RNA samples were extracted from petals, calyx, and stamens of flowers, all at developmental stage S4 (**Figure 1**), and from stems, leaves, and roots. RNA was also extracted from stylopodia containing nectaries from flowers at five different developmental stages (S1, S2, S3, S4, and S5; **Figure 1**). MS flowers secrete nectar from stage 4 until they are pollinated (Zha HG; personal observation). cDNA synthesis was performed according to the manual using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) with oligo(dT) primer. Quantitive PCR was performed with the LightCycler 96 system (Roche Applied Science) and FastStart Essential DNA Green Master (Roche Diagnostics). The PCR conditions were as follows: 94°C for 5 min and then 45 cycles of PCR (95°C for 15 s, 53°C for 15 s and 72°C for 15 s). Gene-specific primers (GulLORTF and GulLORTR for *MsGULLO*; GLDHRTF and GLDHRTR for *MsGLDH*), designed according to the cloned full-length sequences, are listed in **Supplementary Table S1**. The abundance of transcripts was analyzed using the delta delta Ct (ddCt) method based on relative quantification with normalizing to the housekeeping gene: *18S rRNA*. The bean *18S rRNA* specific primers, 18SF and 18SR, were used for the amplification. We also tested the *EF-1α* and *actin* genes to be used as reference genes in qPCR, and

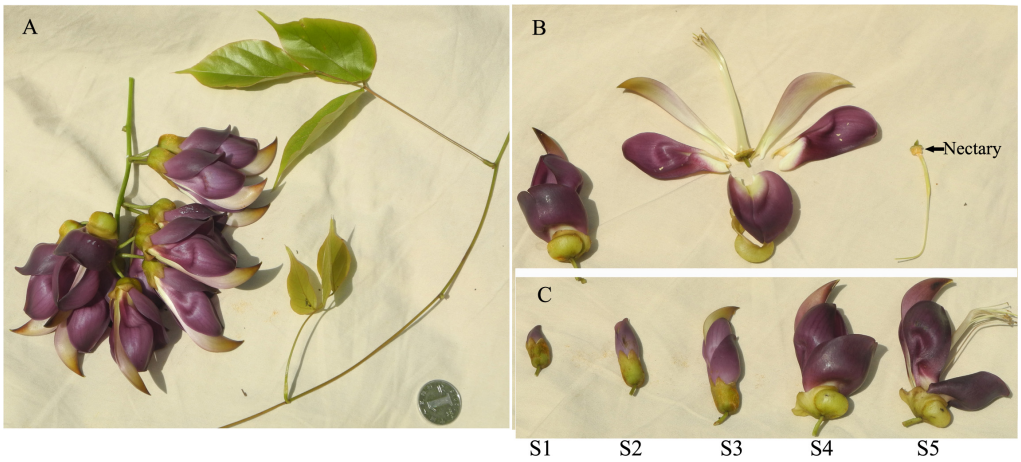

FIGURE 1 | *Mucuna sempervirens* plant. **(A)** Inflorescence, stems and leaves. **(B)** The structure of flower, with nectary indicated by an arrow. **(C)** Different developmental stages of flower. S1 stage, very young flower (corolla less than 1.5 cm long); S2 stage, young flower (corolla ca 2.5 cm long, almost half the length of the adult flower); S3 stage, near adult flower with more than 3 cm long corolla but without enlarged calyx and nectar production; S4 stage, adult flower with nectar in enlarged calyx and keel; S5 stage, old flower with keel opened, and pistil and stamen no longer contained within the keel.

all showed similar expression patterns. All qPCR experiments were repeated with three independent biological replicates and amplicon specificity was checked by high-resolution melting curve analysis.

Bioinformatics Analyses

The theoretical isoelectric point (pI), molecular weight, and hydrophobicity of MsGulLO were calculated using the ProtParam tool available through the ExPasy Web site[1] (Gasteiger et al., 2005). Protein domains were predicted using the Pfam database[2] (Finn et al., 2016). N-terminal signal peptide and cleavage sites were predicted using SignalP 4.1 server[3] (Nielsen, 2017). Predictions of MsGulLO and MsGLDH subcellular localizations were performed by the TargetP webserver[4] (Emanuelsson et al., 2007) and YLoc[5] based on the "YLoc-HighRes Plants model" (Briesemeister et al., 2010).

Phylogenetic Analysis

Twenty six terminal enzymes in the biosynthesis of AsA or its analogs, including both animals and plant sources, were retrieved from Swiss-Prot and used for phylogenetic analysis, including MsGulLO, MsGLDH, plant GulLOs, plant GLDHs, animal GulLOs, ALOs, GulLDH, GalLO, and GUO. Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 was used for the construction of sequence alignments and phylogenetic trees with the amino acid sequences (Kumar et al., 2016). Evolutionary trees were inferred using Maximum Likelihood method. The LG (Le and Gascuel, 2008) model, with invariant sites and gamma distribution (LG + I + G) was estimated as the best-fitting model of amino acid substitution from the data. Bootstrap values were calculated using 1000 replications.

MsGulLO Purification and Enzymatic Assays

MsGulLO was part-purified from raw MS nectar using SEC. Briefly, 20 ml pooled MS floral nectar was used for MsGulLO purification. The proteins in the nectar were concentrated 10 times by ultracentrifugal filtering with Amicon Ultra centrifugal filters (cut-off 10 kDa; Millipore). Two milliliter of the concentrate was then applied onto a Superdex 75 column (60 cm × 1.6 cm) equilibrated in 100 mM sodium acetate buffer, pH 5.0. The column was run at a flow rate of 30 ml h^{-1}, and 1 ml fractions were collected. Protein elution was monitored by A280. Peak fractions were analyzed by SDS-PAGE under non-reducing conditions, and fractions (fraction 26-28; **Supplementary Figure S1**) containing MsGulLO protein of sufficient purity were pooled, and quantified. The isolated protein was run on a denaturing SDS polyacrylamide gel and subjected to mass spectrometry (MALDI-TOF/TOF) to ascertain its purity, then stored at −80°C until further use.

Raw MS nectar and isolated MsGulLO's L-galactono-1,4-lactone dehydrogenase activity, were each tested for their ability to reduce cytochrome C at 550 nm, following Aboobucker et al. (2017). The degree of L-gulono-1,4-lactone oxidase activity in MsGulLO was measured by monitoring AsA production in the reaction using L-GulL as the substrate, following Aboobucker et al. (2017). MsGulLO's oxidase activity was assayed spectrophotometrically using an o-dianisidine-peroxidase coupled assay with L-GulL, L-GalL, D-GluL, glucose, fructose, mannose, sucrose, xylose, arabinose, and AsA as substrates according to Bergmeyer (1974). All assays were performed in triplicates, and the mean ± standard deviations are presented.

[1]http://web.expasy.org/protparam/

[2]http://pfam.xfam.org

[3]http://www.cbs.dtu.dk/services/SignalP/

[4]http://www.cbs.dtu.dk/services/TargetP/

[5]http://abi.inf.uni-tuebingen.de/Services/YLoc/

RESULTS

Mucuna sempervirens Floral Nectar Contains AsA and Hydrogen Peroxide, but Not L-Gulono-1,4-Lactone

Mucuna sempervirens secretes nectar from flower developmental stage S4 (**Figure 1C**) until pollinated; in total ca. 50∼150 μl of nectar per flower. MS nectar was acidic with a pH value of 5.3 ± 0.2 and a total sugar concentration of 25.0 ± 5.2 Brix°(mean ± SD, n = 15). HPLC showed that MS nectar in this study was a sucrose rich type, containing sucrose, glucose, and fructose at a ratio of 1: 0.22: 0.32. L-gulono-1,4-lactone was not detected in MS nectar by HPLC using refractive index detection. The concentration of hydrogen peroxide detected in MS nectar was 62.1 ± 10.5 μM (mean ± SD, n = 15). The concentration of total and reduced AsA in MS nectar were 4.3 ± 0.5 and 2.4 ± 0.4 μM (mean ± SD, n = 6), respectively. Only oxidized GSH (GSSG) was detected in MS nectar, and this had a concentration of 0.74 ± 0.07 μM (mean ± SD, n = 6). The mean concentration of protein in MS nectar was 370 μg ml^{-1} (n = 15) which was almost ten times higher than reported *Canavalia gladiata* and *Nicotiana tabacum* nectar protein concentration and didn't need to be concentrated before used for gel electrophoresis analysis (Zha et al., 2012; Liu et al., 2013; Ma et al., 2017).

A Plant L-Gulonolactone Oxidase Homolog Was Detected in MS Nectar

To identify MS nectar proteins, we performed 2D gel electrophoresis of MS nectar, which yielded more than 10 spots in the gel after visualization by Coomassie Brilliant Blue G-250 and silver staining (**Figure 2**). As we previously reported, most of the MS nectar proteins were alkaline, and ranged in molecular mass from 17 to 100 kDa (Zha et al., 2013). All visible protein spots (19 in total) were subjected to tryptic digestion and then analyzed by MALDI-TOF/TOF. Because proteins might be truncated or modified during the process of 2-DE, different spots in the gel could represent the same protein. In this study, three proteins were successfully identified by mass spectrometry (MALDI TOF/TOF): L-gulonolactone oxidase, a desiccation-related protein, and a pathogenesis-related protein 1-like protein (**Figure 2** and **Supplementary Table S2**). From the first three spots, two peptides, QEDAIDFDITYYR (MW 1648.67) and LYEDIIEEVEQLGIFK (MW 1937.91), were identified; these matched the identity of L-gulonolactone oxidases from *Glycine max* (accession no. XP_006604910), *Cajanus cajan* (accession no. XP_020213315), and *Malus domestic* (accession no. XP_008353193). Therefore, the protein identified as an L-gulonolactone oxidase in MS nectar was designated as MsGulLO.

MsGulLO cDNA Cloning and Amino Acid Sequence Analysis

Using a combination of 5′ and 3′RACE methods, full-length cDNAs encoding MsGulLO and MsGLDH were cloned (accession numbers MF327592 and MG021324, respectively).

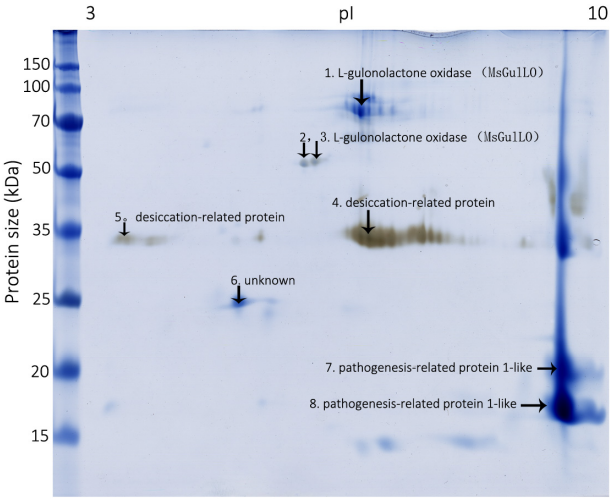

FIGURE 2 | *Mucuna sempervirens* nectarines on 2D gel. Identified nectar proteins on the gel, including MsGulLO, are indicated by arrows.

The *MsGulLO* gene consists of 1722 bp, and encodes a protein of 573 amino acids. In a BLAST search, this gene showed high identity with reported plant GulLOs, with the highest identity (90%) with the sequence of *Glycine max* L-gulonolactone oxidase-4 (accession no. XM_006604847). However, the closest match with a well functional characterized animal GulLO (accession no. P10867 from *Rattus norvegicus*) was only 27%. The full amino acid sequence of MsGulLO was deduced from the cDNA sequence, and subjected to a Pfam search which revealed an ALO family domain at positions 375–520 (E-value: $5.6e^{-13}$) and an FAD binding domain at positions 2–133 (E-value: $6.3e^{-22}$). However, in common with other plant GulLOs, MsGulLO doesn't have a FAD-binding motif in its N terminus (Leferink et al., 2008; Aboobucker and Lorence, 2016). The mature MsGulLO protein had a neutral isoelectric point of 6.72, and a predicted molecular mass of 61,962.52 Da which is consistent with the 2-DE results (**Figure 2**). The first 18 amino acids of MsGulLO were predicted to form a signal peptide by SignalP, which suggested that MsGulLO is a secreted protein. In addition, TargetP and YLoc predicted that MsGulLO would enter the secretory pathway, and become located in extracellular space. Mature MsGulLO is predicted to be a stable and hydrophilic protein, with an instability index (II) of 30.23, and a grand average of hydropathicity (GRAVY) score of −0.315. These predictions are in agreement with the hypothesis that MsGulLO is secreted out from the nectary, and presents as a soluble protein in nectar.

The mass spectrometric data of MsGulLO confirmed the presence of 17 peptides that matched the predicted masses derived from the translated sequence of the *MsGulLO* gene (**Supplementary Table S3**). These peptides covered 36.8% of the total amino acid sequence of the mature MsGulLO protein. Thus, we conclude that the *MsGulLO* gene encodes a plant L-gulono-1,4-lactone oxidase homolog, MsGulLO.

The *MsGLDH* gene consists of 1752 bp, and encodes a protein of 583 amino acids. The sequence of the *MsGLDH*

gene showed high identity with reported plant GLDHs, with the highest identity (93%) being to the sequence of *Cajanus cajan* L-galactono-1,4-lactone dehydrogenase (accession no. XM_020367752). The MsGLDH protein was alkaline (theoretical pI: 8.46) and had a predicted molecular mass of 66,171.67 Da. Two ALO family domains at positions 255–343 and 370–576 (*E*-value: $8.5e^{-06}$ and $8.8e^{-12}$), and an FAD binding domain at positions 4–135 (*E*-value: $2.5e^{-28}$), were detected in this MsGLDH protein sequence by Pfam search. However, unlike MsGulLO, MsGLDH was predicted to be a mitochondrial protein, containing no signal peptide but a FAD-binding motif in the N terminus. This prediction is in agreement with plant GLDHs being localized in the mitochondria (Aboobucker and Lorence, 2016). MsGLDH had an instability index (II) of 44.97, which indicated that it is not theoretically stable.

MsGulLO and Other Plant GulLOs Are Divergent From Other Aldonolactone Oxidoreductases

From the protein sequences available, an unrooted maximum likelihood phylogenetic tree was produced (**Figure 3**). It showed that those aldonolactone oxidoreductases that function as terminal enzymes in the biosynthesis of AsA in different organisms formed two distantly related clades. MsGulLO was grouped with other plant GulLOs with strong support values. Plant GLDHs, animal GulLOs, ALOs, GulLDH, GalLO, and GUO from other organisms formed another clade. Based on this, we speculated that so-called plant GulLOs have functions that are distinct from animal GulLOs and other well-established terminal enzymes in AsA biosynthesis. All seven GulLOs from *Arabidopsis thaliana* (AtGulLOs) were incorporated in this analysis and MsGulLO was closely related to the clade formed by AtGulLO 2, 5, and 6. Transgenic analysis suggested that AtGulLO 5 played roles in AsA biosynthesis (Maruta et al., 2010; Aboobucker et al., 2017), which indicates that MsGulLO probably has a similar function.

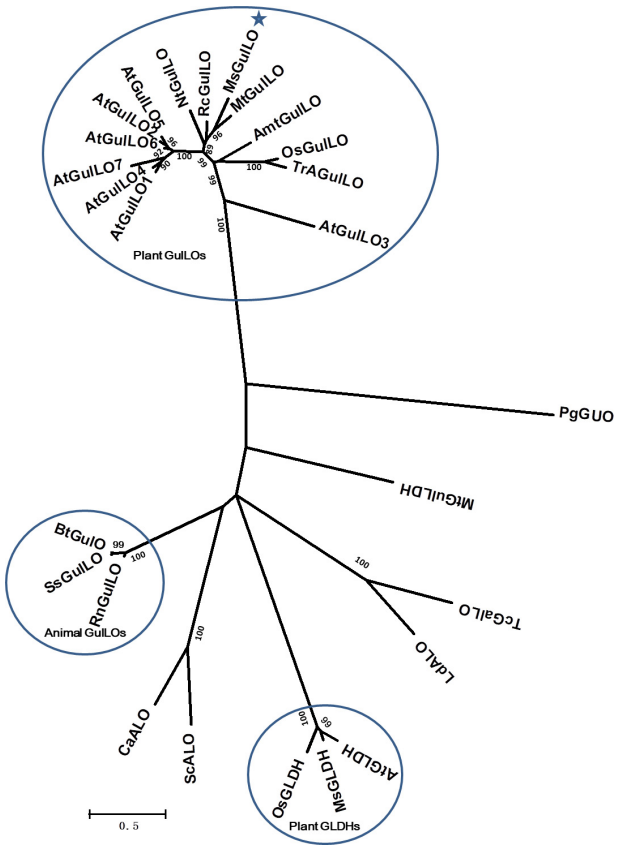

FIGURE 3 | Phylogenetic analysis of 26 terminal enzymes that are involved in the biosynthesis of AsA or its analogs. The phylogenetic relationship was reconstructed by using the Maximum Likelihood method and the Le and Gascuel (2008) model by Mega 7.0. MsGulLO is indicated by a star. Plant GulLOs, plant GLDHs, and animal GulLOs are circled. Numbers on branches indicate the bootstrap percentage values (>80%) calculated from 1000 bootstrap replicates. The species used in the unrooted phylogeny tree construction are as follows, with names followed by accession number. GulLOs from plants: *Amborella trichopoda* (AmtGulLO; W1PKS5), *Arabidopsis thaliana* (AtGulO1 to AtGulO7; Q9C614, Q6NQ66, Q9LYD8, Q9FM82, O81030, O81032, and Q9FM84), *Medicago truncatula* (MtGulLO; A0A072TYF3), *Mucuna sempervirens* (MsGulLO; A0A290U7F5), *Nicotiana tabacum* (NtGulLO; A0A1S4B1A6), *Oryza sativa* (OsGulLO; Q10I64), *Ricinus communis* (RcGulLO; B9SVF9), *Triticum aestivum* (TrAGulLO; A0A077RZP9); GulLOs from animals: *Bos taurus* (BtGulLO; Q3ZC33), *Rattus norvegicus* (RnGulLO; P10867), *Sus scrofa* (SsGulO; Q8HXW0); GLDHs from *Arabidopsis thaliana* (AtGLDH; Q9SU56), *Mucuna sempervirens* (MsGLDH; AVM41577), *Oryza sativa* (OsGLDH; Q2QXY1); ALOs from *Candida albicans* (CaALO; O93852), *Leishmania donovani* (LdALO; C8CCV9), *Saccharomyces cerevisiae* (ScALO; P54783). GulLDH from *Mycobacterium tuberculosis* (MtGulLDH; P9WIT3). GalLO from *Trypanosoma cruzi* (TcGalLO; Q4DPZ5). GUO from *Penicillium griseoroseum* (PgGUO; Q671X8).

MsGulLO Is Mainly Expressed in the MS Nectary

Expression analysis of the *MsGulLO* gene in MS leaf, stem, root, petal, stamen, and nectaries at five developmental stages was accomplished by qRT–PCR. The relative expression level of *MsGulLO* was high in nectaries at developmental stage 3, 4, and 5 (**Figure 4A**). *MsGulLO* transcripts were also detected in petal, stamen, and nectary at developmental stage 2, and in much lower quantities in the stem; they were not detected in the root, leaf, petal, or nectary at developmental stage 1. Our results indicate that *MsGulLO* is mainly expressed in flowers, and especially in the nectary. *MsGulLO* gene transcripts start to accumulate in the nectary ahead of blooming, and before nectar secretion, which demonstrates that it is synthesized before these things happen.

However, *MsGLDH* showed a completely different expression pattern to *MsGulLO* (**Figure 4B**). *MsGLDH* transcripts were detected in all the tissues tested in this study. Unlike MsGulLO, the relative expression level of MsGLDH was low in nectaries at developmental stage 3, 4, and 5, but high in developmental stage 1 and 2 (**Figure 4B**). Therefore, *MsGLDH* is shown to be constitutively expressed and its function might not be related with nectary or flower development. It is consistent with this that no MsGLDH was detected in MS nectar in this study.

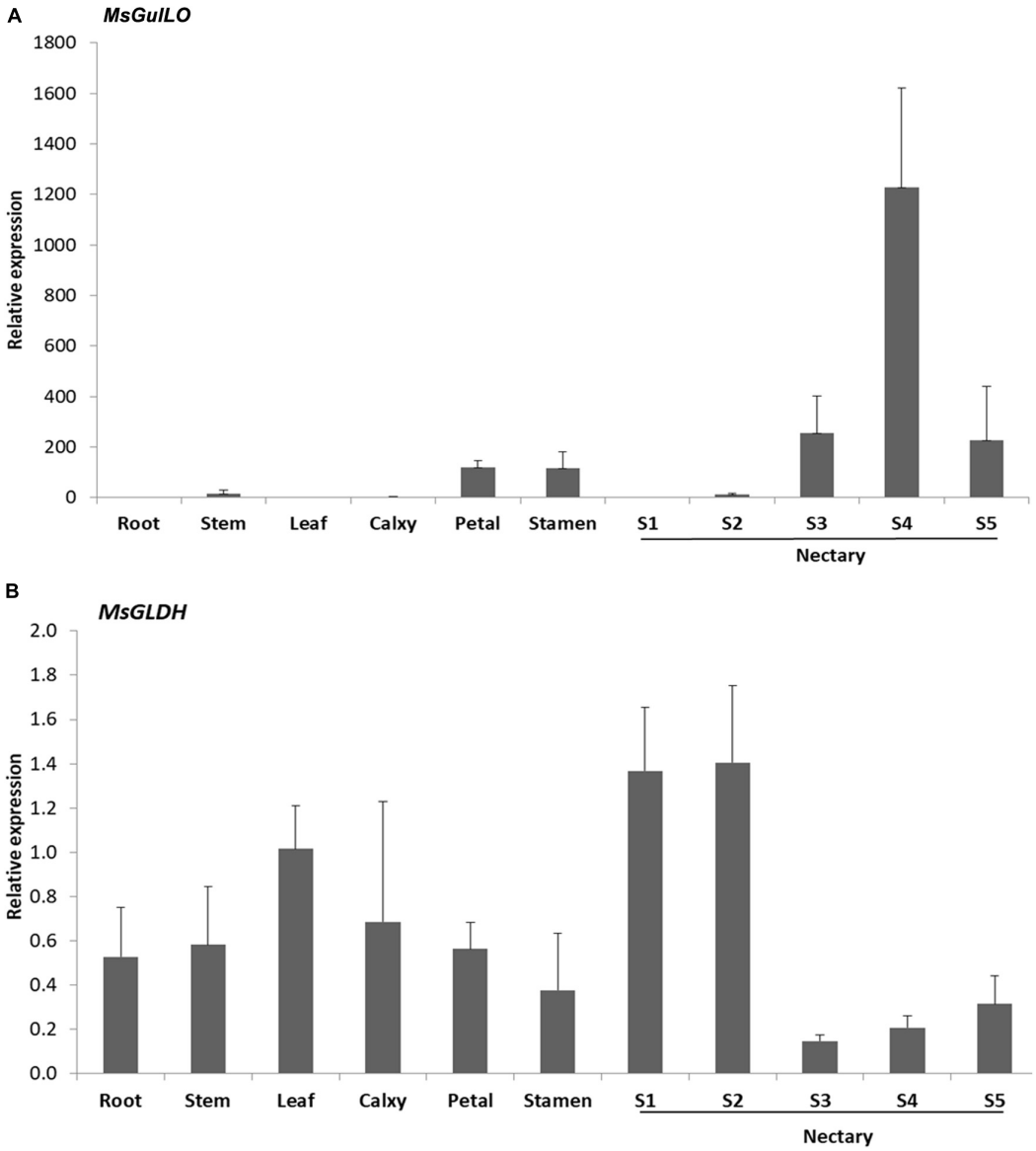

FIGURE 4 | Determination by qRT-PCR of transcript levels of *MsGulLO and MsGLDH* in various tissues. **(A)** *MsGulLO* expression patterns. **(B)** *MsGLDH* expression patterns. Whole RNA was isolated from the following tissues: stems, roots, petals, calyxes, stamens, leaves; S1–S5, nectaries at developmental stage 1–5. All data were normalized to 18S rRNA transcript levels. Values represent mean ± SE from three biological repeats.

MsGulLO Had No L-Gulono-1,4-Lactone Oxidase or L-Gulono-1,4-Lactone Dehydrogenase Activity in AsA Biosynthesis

In this study, MsGulLO was isolated from MS nectar proteins and other nectar components, such as sugars, using SEC, and the elution profile of MS nectar proteins is depicted in **Supplementary Figure S1**. MsGulLO containing fractions (no. 26–28) were pooled for subsequent analysis (**Supplementary Figure S1**). The isolated MsGulLO migrated as one major band in SDS-PAGE gel with a MW of 70 kDa with several very weak bands (**Figure 5**). The mass spectrometric peptide fingerprinting analysis proved that the part-purified protein was identical to the MsGulLO protein identified from 2-DE gel (data not shown).

Both raw MS nectar and isolated MsGulLO showed no GLDH and GulLO activity as animal GulLOs, and no AsA was generated during the assay; this is consistent with purified recombinant AtGulLO5 having no GulLO activity (Aboobucker et al., 2017). AtGulLO5 was demonstrated to have GLDH activity (Aboobucker et al., 2017), but this study showed no GLDH activity for MsGulLO. Using an o-dianisidine-peroxidase coupled assay, MsGulLO showed weak oxidase activity toward L-GulL (0.08 ± 0.02 units mg^{-1}), L-GalL (0.10 ± 0.02), D-GluL (0.08 ± 0.02), glucose (0.08 ± 0.02), and fructose (0.06 ± 0.01) (mean ± SD, n = 3 in each case).

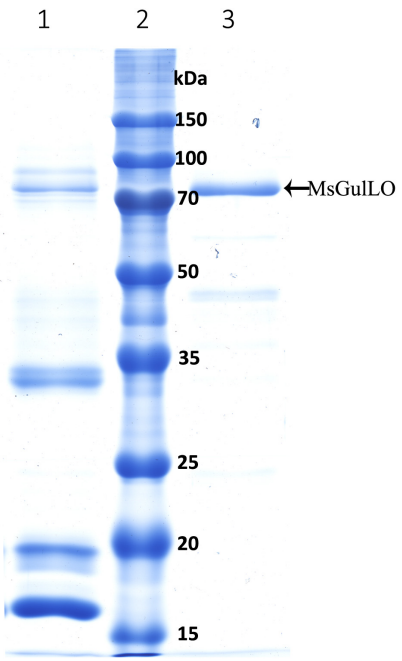

FIGURE 5 | SDS–PAGE of *M. sempervirens* nectar proteins and isolated MsGulLO. Lane 1, MS nectar proteins; lane 2 molecular weight markers; lane 3, Isolated MsGulLO.

L-GulL, L-GalL, and D-GluL were not detected in MS nectar by using HPLC with refractive index detection. MsGulLO showed no oxidase activity to raffinose, mannose, sucrose, arabinose, and AsA even though both sucrose and AsA were present in MS nectar. Therefore, both glucose and fructose are probably MsGulLO's natural substrates.

DISCUSSION

AsA (vitamin C) is an enzyme co-factor in eukaryotes that also plays an important role in protecting photosynthetic eukaryotes against damaging ROS derived from the chloroplast (Wheeler et al., 2015). In many animal lineages, L-gulonolactone oxidase (GulLO) is the terminal enzyme in the AsA biosynthetic "animal" pathway. Growing molecular and biochemical evidence from photosynthetic eukaryote lineages has demonstrated an alternative "plant" pathway, also called the "Smirnoff-Wheeler pathway," in which GulLO is functionally replaced with GLDH (Wheeler et al., 2015). Another AsA biosynthetic pathway existing in plants has been suggested in which the oxidation of L-GulL to AsA is the final step, and GulLO is the terminal enzyme (Maruta et al., 2010; Aboobucker et al., 2017). An enzyme family in plants, which includes AtGulLO1∼7 from *A. thaliana*, was reported to exhibit some similarity to animal GulLO (Maruta et al., 2010). Therefore, detailed characterization of the plant GulLOs is important, but it remains very rare. Maruta et al. (2010) reported that overexpression of AtGulLO 2, 3, or 5 in tobacco cell lines could result in increased AsA levels after L-GuL feeding. However, they failed to obtain the recombinant protein and test the enzymatic activity directly. Until recently, the recombinant AtGulLO5 was firstly isolated and characterized *in vitro* and demonstrated to be not an oxidase but a dehydrogenase which could convert L-GulL to AsA with an absolute specificity for L-GulL (Aboobucker et al., 2017). This investigation also demonstrated that AtGulLO5 is different from the existing plant GLDHs (specific to L-GalL) or mammalian GulLOs.

Here we describe the characterization of an AtGulLO5 homolog, MsGulLO, from the legume *Mucuna sempervirens* (MS), achieved by direct protein purification, enzymatic assays, gene cloning, and expression analysis. Our data did not support the hypothesis that MsGulLO and its plant homologs are the terminal enzyme in the suggested alternative plant AsA biosynthetic pathway. First, no L-gulonolactone oxidase or dehydrogenase activity was detected. Adding L-GulL into MS nectar or isolated MsGulLO did not result in any detectable AsA generation, which likewise indicates that MsGulLO can't convert L-GulL to AsA (data not shown). In addition, no L-GulL could be detected in MS nectar. Secondly, it is known that flavin plays essential roles in both animal GulLO and plant GLDH activity (Smirnoff, 2001). However, MsGulLO is not a flavin-containing protein, and no flavin could be detected in MS nectar by fluorescence analysis. The presence or absence of FAD from the system had no effect on MsGulLO's oxidase activity (data not shown). The FAD-binding motif is not present in the protein sequence of MsGulLO, AtGulLOs, or other so-called plant GulLOs (Leferink et al., 2008; Aboobucker and Lorence, 2016). This indicated that plant GulLOs might have different activity and/or a distinct catalysis mechanism from animal GulLOs. Thirdly, all plant GulLOs including MsGulLO and AtGulLOs were predicted to be secretory proteins with a predicted signal peptide. Our finding confirmed this prediction because MsGulLO is secreted into nectar and mainly expressed in the flower and nectary. However, animal GulLOs and plant GLDHs are not secretory proteins (Wheeler et al., 2015). Plant GLDHs are located in mitochondria, and have a different destination from plant GulLOs. This also suggests that Plant GLDHs and GulLOs carry out different functions in plants. Fourthly, even though plant GulLOs share high sequence similarity with each other, the identity between plant GulLOs and animal GulLOs is very low, less than 30% (Aboobucker and Lorence, 2016). Phylogenetic analysis also showed that plant GLDHs are far closer to animal GulLOs than either are to plant GulLOs, which indicates that plant GulLOs probably have a different evolutionary origin to either, and perform a different physiological function. Fifthly, AsA is mostly produced and accumulates in photosynthetic organs in land plants, such as leaves (Gest et al., 2013). However, in this study, no *MsGulLO* transcripts were detected in leaves. Therefore, MsGulLO looks unlikely to be involved in AsA biosynthesis.

The true function of MsGulLO in MS nectar remains unclear. Isolated MsGulLO did show a weak glucose and fructose oxidase activity, which could produce hydrogen peroxide using glucose and fructose as the substrate. High concentrations of hydrogen peroxide in nectar has been deemed to protect the nectary from microorganism growth (Carter and Thornburg, 2004a;

Nocentini et al., 2015; Roy et al., 2017). To our knowledge, nectarin V (NEC5) from tobacco plants is the only protein exhibiting glucose oxidase activity that has been identified in nectar, and NEC5 is a flavin-containing berberine bridge enzyme-like protein (Carter and Thornburg, 2004b). With the high concentration of simple sugars present in tobacco nectar, the likely function of NEC5 was to generate the antimicrobial levels of hydrogen peroxide found therein; it hence plays an important role in the "nectar redox cycle"(Carter and Thornburg, 2004a). Both MsGulLO and NEC5 have glucose oxidase activity, but MsGulLO didn't require FAD for its oxidase activity. We determined that glucose, fructose and hydrogen peroxide all coexist in MS nectar even though the concentration of hydrogen peroxide was not as high as in reported tobacco nectar (Carter and Thornburg, 2004a). Because the concentration of AsA was ten times less than that of hydrogen peroxide in MS nectar, it is doubtful that AsA could detoxify hydrogen peroxide in the nectar. We also noticed that the concentration of hydrogen peroxide in individual MS nectar samples from different flowers could vary dramatically. It looks like that the generation of hydrogen peroxide in nectar might be triggered by some external stimulus, such as microorganisms introduced by pollinators or wind, and that it is under rapid regulation. In addition, MsGulLO has no ascorbate peroxidase activity, and we found that it could not produce hydrogen peroxide using AsA as the substrate (data not shown). Thus, we suggest that MsGulLO might function in the generation of hydrogen peroxide in nectar using glucose and fructose as substrate. However, the mechanism regulating hydrogen peroxide metabolism in nectar is still unknown, and hence requires further investigations. Even though AsA was detected in MS nectar, we couldn't find any evidence to link MsGulLO's activity with the generation of AsA.

CONCLUSION

In this study, an L-gulonolactone oxidase like protein (MsGulLO) was identified in the floral nectar from MS (Fabaceae) by 2-DE and mass spectrometry. The full length *MsGulLO* cDNA was cloned, and found to encode a protein of 573 amino acids with a predicted signal peptide; it was hence predicted to enter the secretory pathway. MsGulLO has high similarity to other plant GulLOs, such as AtGulLO5 in *A. thaliana* which was suggested to be involved in the pathway of L-AsA biosynthesis. Phylogenetic analysis shows that MsGulLO and plant GulLOs are divergent from animal L-gulonolactone oxidases, whose functions are well characterized. MsGulLO was a secreted protein and expressed only in flowers and especially in nectary before blooming. However, cloning and gene expression analysis showed that MsGLDH, a validated vital enzyme in plant AsA biosynthesis, was located in the mitochondria, whereas it is expressed in flowers, roots, stems, and especially leaves. MsGulLO was purified to near homogeneity from raw MS nectar by gel filtration chromatography. The enzyme was determined to be a neutral monomeric protein with an apparent molecular mass of 70 kDa. MsGulLO is not a flavin-containing protein and has no regular GulLO or GLDH activity, however, it does have oxidase activity using glucose and fructose as natural substrates. MsGulLO is suggested to function in maintaining the homeostasis of hydrogen peroxide in nectar but to not be involved with AsA biosynthesis in nectar or other tissues in the plant.

AUTHOR CONTRIBUTIONS

H-GZ, H-XZ, and HS conceived and designed the research. X-LM, Y-QS, and H-XZ conducted the experiments. J-YF and RM analyzed the data. RM and H-XZ wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01109/full#supplementary-material

FIGURE S1 | Elution profile of MsGulLO on a Superdex-75 size exclusion chromatography column.

TABLE S1 | Primers used for *MsGulLO* and *MsGLDH* cDNA cloning and qPCR.

TABLE S2 | *Mucuna sempervirens* nectar proteins identified by 2-DE and mass spectrometry.

TABLE S3 | MsGulLO peptides identified by mass spectrometry (MALDI-TOF/TOF).

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frontiers in Plant Science

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Nectar Sugar Modulation and Cell Wall Invertases in the Nectaries of Day- and Night- Flowering *Nicotiana*

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Nectar composition varies between species, depending on flowering time and pollinator type, among others. Various models of the biochemical and molecular mechanisms underlying nectar production and secretion have been proposed. To gain insights into these mechanisms, day- and night-flowering tobacco (*Nicotiana*) species with high or low proportions of hexoses in the nectar were analyzed. Nectar and nectaries were simultaneously collected, throughout the day and night. Soluble sugars and starch were determined and the activity and expression level of cell wall invertase (CW-INVs) were measured in nectaries. Nectaries and nectar of the five *Nicotiana* species contained different amounts of sucrose, glucose, and fructose. CW-INV activity was detected in the nectaries of all *Nicotiana* species and is probably involved in the hydrolysis of sucrose in the nectary tissue and during nectar secretion. The larger differences in the sucrose-to-hexose-ratio between nectaries and nectar in diurnal species compared to nocturnal species can be explained by higher sucrose cleavage within the nectaries in night-flowering species, and during secretion in day-flowering species. However, cell wall invertase alone cannot be responsible for the differences in sugar concentrations. Within the nectaries of the *Nicotiana* species, a portion of the sugars is transiently stored as starch. In general, night-flowering species showed higher starch contents in the nectaries compared to day-flowering species. Moreover, in night flowering species, the starch content decreased during the first half of the dark period, when nectar production peaks. The sucrose concentrations in the cytoplasm of nectarial cells were extrapolated from nectary sucrose contents. In day-flowering species, the sucrose concentration in the nectary cytoplasm was about twice as high as in nectar, whereas in night-flowering species the situation was the opposite, which implies different secretion mechanisms. The secreted nectar sugars remained stable for the complete flower opening period, which indicates that post-secretory modification is unlikely. On the basis of these results, we present an adapted model of the mechanisms underlying the secretion of nectar sugars in day- and night-flowering *Nicotiana*.

Keywords: floral nectar, nectaries, cell wall invertase, tobacco, *Nicotiana*, sugar composition, diurnal/nocturnal

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INTRODUCTION

Nectar is a sugar-rich solution which is produced by most angiosperm plants to fulfill extensive functions, e.g., the attraction of pollinators and protection against herbivores (Brandenburg et al., 2009; González-Teuber and Heil, 2009; Adler et al., 2012). Nectar is produced by and secreted from nectaries, which are highly specialized glands, and the surrounding tissue. In *Nicotiana,* all floral

nectaries are located at the basal side of the gynoecium (Bernardello, 2007) and during nectary development, β-carotene is expressed, which results in an orange coloring (Horner et al., 2007). The synthesis and secretion of floral nectar has been the subject of several studies, and different models on the biochemical and molecular mechanisms underlying nectar secretion have been proposed (Ge et al., 2000; Horner et al., 2007; Kram et al., 2009; Mosti et al., 2013; Stpiczyńska et al., 2014). But due to the enormous diversity of flowering plants, there are still several variables that warrant further study (Roy et al., 2017). A very basic theory of nectar secretion proposes an apoplastic movement of metabolites from the phloem to the nectary surface (Vassilyev, 2010). However, the metabolite composition differs between the phloem sap and the nectar (Lohaus and Schwerdtfeger, 2014), which does not support the proposed apoplastic method of nectar secretion. Other hypotheses propose that various enzymes and transport proteins are involved in nectar production. For certain plant species, an eccrine secretion mode has been proposed, wherein sucrose is delivered from the phloem to the nectary parenchyma cells, and there the sucrose is transiently converted to starch or exported to the apoplast directly. A plasma membrane-localized sucrose transporter SWEET9 is essential for this transport (Lin et al., 2014). SWEET9 functions as a facilitated diffusion transporter for sucrose, and mutants lacking SWEET9 do not produce nectar, e.g., in *Nicotiana attenuata* (Lin et al., 2014). Once sucrose is exported from the nectary, it is then hydrolysed by an extracellular cell wall invertase (CW-INV) into glucose and fructose (Ruhlmann et al., 2010). In a third proposed secretory mechanism, nectar metabolites are transported symplastically to the outer nectary cells and then packed into vesicles, which are produced by the endoplasmic reticulum (ER) or the Golgi complex, to fuse with the plasma membrane and release the nectar metabolites to the nectary surface (Fahn, 1979a,b). These three models for nectar secretion are not necessarily mutually exclusive, and other modes of nectar secretion can occur in different plant species.

In some plant species, starch accumulates in the nectaries and peaks approximately 24 h before anthesis and then declines rapidly, which is the basis for the hypothesis that starch is one source of sugars for nectar production before and during nectar secretion (Nepi et al., 1996; Horner et al., 2007; Ren et al., 2007a,b). Genes encoding anabolic enzymes involved in starch synthesis were found to be more highly expressed at the early stages of nectary development, and genes encoding catabolic enzymes were expressed at later stages (Ren et al., 2007a). However, studies on lychee (*Litchi chinensis*) floral nectaries have shown that the nectar sugar is composed of both phloem sap and products of starch degradation in the nectaries (Ning et al., 2017).

Of the sugars found in nectar, the most prevalent are sucrose and the hexoses glucose and fructose (Percival, 1961; Baker and Baker, 1983; Tiedge and Lohaus, 2017). Given that hexoses are typically not components of the phloem sap (Lohaus and Schwerdtfeger, 2014), the proportion of hexoses in nectar depends on the presence and activity of sucrose-cleaving enzymes. Sucrose cleavage in plants can be catalyzed by at least two types of enzymes: reversible sucrose cleavage is catalyzed by sucrose synthase (SuS; EC 2.4.1.13), a glycosyltransferase; and irreversible sucrose cleavage is catalyzed by invertases, which catalyze hydrolysis (β-fructofuranosidases; EC 3.2.1.26). Invertases exist in numerous isoforms with various subcellular localizations and biochemical properties (Roitsch and González, 2004). These enzymes can be classified into three groups: vacuolar invertases (V-INVs), extracellular invertases (CW-INVs), and neutral invertases (N-INVs). Whereas N-INVs have an alkaline pH-optimum, V-INVs and CW-INVs are so-called "acidic invertases" because they work most efficiently between pH 4.5 and 5.0. Extracellular invertases are non-soluble proteins that are ionically bound to the cell wall (Sturm, 1999). A separate gene encodes for each of the isoforms, which have a high identity and share common features, e.g., the pentapeptide NDPNG (βF-motif) close to the N-terminus of the mature protein, and WECXDF, an amino acid sequence closer to the C-terminus (Sturm and Chrispeels, 1990; Roitsch and González, 2004).

For some plant species, e.g., carrot (*Daucus carota*) and tomato (*Solanum lycopersicum*), different organ- and development-stage-specific expression patterns of acid invertase were shown (Sturm et al., 1995; Godt and Roitsch, 1997). Usually, invertase expression is increased in rapidly growing tissues with a high demand for hexoses (Weschke et al., 2003). Interestingly, for both carrot and tomato, the mRNA expression of an acidic invertase was found to be specific to flowers and flower buds (Lorenz et al., 1995; Godt and Roitsch, 1997). It was assumed that this flower-specific extracellular invertase is essential for male and female organ development, e.g., to supply the anthers with carbohydrates (Dorion et al., 1996; Godt and Roitsch, 1997). More recently, it was shown that CW-INV is also crucial for nectar secretion in *Arabidopsis* (Ruhlmann et al., 2010). AtCWINV4 expression was found to be highly up regulated in nectaries of *A. thaliana* compared to other tissues (Kram et al., 2009). Furthermore, two independent *cwinv4*-mutant lines with greatly diminished activity of total CW-INV in whole *Arabidopsis* flowers secreted no nectar, although the nectary ultrastructure appeared to be similar to that of wild-type plants (Ruhlmann et al., 2010).

The genus *Nicotiana* is highly diverse in terms of flower morphology and pollination mode. In a study involving 20 *Nicotiana* species, the sugar concentration in the nectar of several day- and night flowering species was measured (Tiedge and Lohaus, 2017). The genus *Nicotiana* contains species with sucrose-rich nectars as well as hexose-rich nectars, and the exact nectar composition depends on the pollinator type, flowering time, corolla length and other environmental factors (Tiedge and Lohaus, 2017). The sucrose-to-hexose ratio ranged from 0.1 to 2.0 and was fairly consistent within a given species.

This finding raises the question of whether the sugar composition in nectar is a result of the sugar composition in the nectaries. Alternatively, a lower sucrose content in nectar could reflect higher invertase activity in the nectaries and during nectar secretion. Furthermore, we aimed to investigate potential differences in invertase expression and activity over the course of a day, in consideration of flower opening and nectar production times. In addition to these pre-secretory and secretory processes, post-secretory processes could also be responsible for varying

sugar composition. In such a scenario, the nectar itself must contain sugar cleaving enzymes.

To further investigate the mechanism underlying nectar production and secretion, five tobacco species with varying properties were examined. Two day-flowering species (*N. tabacum* and *N. africana*) as well as two night-flowering species (*N. sylvestris* and *N. benthamiana*) were included. Within each category (day- or night-flowering), one species had a high sucrose content and one species had a low sucrose content (**Figure 1**). For reproduction, these species rely on pollination either by diurnal birds (*N. africana*: sunbirds; *N. tabacum*: hummingbirds), nocturnal moths (*N. sylvestris*), or otherwise the species is primarily autogamous (*N. benthamiana*) (Tiedge and Lohaus, 2017). Additionally, *N. attenuata* was chosen, which opens its flowers at twilight both in the evening and in the morning and is therefore less dependent on a specific pollinator (Kessler and Baldwin, 2007). To investigate whether the nectar sugar content primarily depends on pre-secretory processes, the secretion process, or post-secretional modification, nectar sugars were compared to nectary sugars at multiple time points per day; additionally, the invertase activity and expression were measured, and post-secretional activity was recorded.

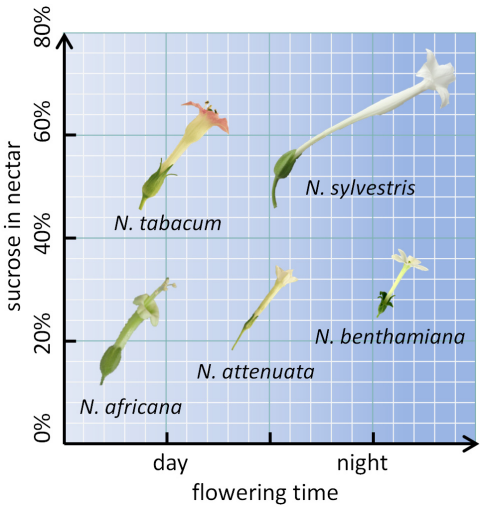

FIGURE 1 | *Nicotiana* species arranged by flowering time and sucrose content. The flower sizes are depicted to relative scale.

MATERIALS AND METHODS

Plant Material

Nicotiana attenuata seeds were provided by the Max Planck Institute for Chemical Ecology (Jena, Germany), *N. benthamiana* seeds were provided by the University of Rostock (Germany), *N. africana* and *N. sylvestris* seeds were provided by the Botanical Garden of Ruhr University Bochum (Germany), and *N. tabacum* seeds were provided by NiCoTa (Rheinstetten, Germany). Each plant was potted in a single 5-L pot with compost soil and grown in a greenhouse at the University of Wuppertal. Cultivation was carried out with a 14-h-light/10-h-dark cycle, an irradiance of approximately 300 μmol photons m^{-2} s^{-1} and a temperature regime of 25°C day/18°C night.

Collection of Nectaries and Nectar

Each sample (∼100 mg) of nectary tissue comprised 20–50 nectaries, depending on the species. At each time point (2 p.m., 8 p.m., 2 a.m., and 8 a.m.), three samples were taken. To collect the nectaries, the gynoecia were extracted from the flowers, and the nectary tissue was dissected with a scalpel and rinsed with ultrapure water to remove external sugars. All samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. The weight of a single nectary was calculated as follows:

$$\textit{Weight per floral nectary}\,[mg] = 100\,mg\,/\,\textit{number of nectaries collected per sample}$$

For each species, at least three nectar samples were taken from three plants at all four time points. The nectar samples were collected with micropipettes, assayed for microbial contamination according to Tiedge and Lohaus (2017) and stored at −80°C until further analysis. In addition, nectar samples were also analyzed by light-microscopy to exclude contamination with pollen. The nectar samples used for post-secretional experiments were left at room temperature for 12, 24, and 48 h. The water content of the nectaries and leaves was determined by drying and weighing those tissues. The following calculation was used:

$$\textit{Water content} = 1 - (\text{dry weight [mg]} / \text{fresh weight [mg]})$$

Analysis of Sugars and Starch in Nectaries and Nectar

For the extraction of soluble metabolites from nectary tissue, a chloroform-methanol-water extraction was performed (Nadwodnik and Lohaus, 2008). The analysis of sugars in nectar, nectaries and leaves via HPLC was conducted according to Lohaus and Schwerdtfeger (2014). Nectar was filtered (0.2 μm nitrocellulose; Schleicher and Schuell, Germany) before HPLC measurements to exclude contamination with pollen. An ion exchange column (CarbopacTM PA10 4 mm × 250mm; Dionex Corp, Sunnyvale, CA, United States) was eluted isocratically with 80 mM NaOH (JT Baker Chemicals). Sugars were detected with a pulse amperometric detector with a gold electrode (ESA Model 5200, Coulochem II, Bedford, MA, United States). The pulse setting was 50, 700, and −800 mV for 400, 540 and 540 ms, accordingly. For external calibration, sugar standards (Sigma-Aldrich, Germany) were measured in parallel. The evaluation of the chromatograms was performed with an integration program (Peaknet version 5.1, Dionex). Starch content of nectaries was determined according to a modified protocol from Riens et al. (1994).

Expression of CWINV

RNA from approximately 50 mg of nectariferous tissue was isolated using a modified protocol from Logemann et al. (1987),

where cetyltrimethylammonium bromide (CTAB) is used to inactivate RNase activity and to form a complex with RNA without adding guanidine. Synthesis of cDNA was performed using the RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Sankt Leon-Rot, Germany) with oligo(dT)$_{18}$ primers. Degenerated primers were designed to amplify CW-INV sequences of the different *Nicotiana* species. The obtained sequences were cloned with the pGEM®-T Easy Vector System (Promega Corporation, Madison, IW, United States) for sequencing, and suitable specific primers for quantitative real-time polymerase-chain-reaction (qRT-PCR) were selected. For verification of the obtained primers and sequences, amplification with proof read polymerase (Phusion High-Fidelity DNA-Polymerase, Thermo Fisher Scientific, Waltham, MA, United States) and blasting with known sequences from NCBI (National Center for Biotechnology Information, Bethesda, MD, United States) was performed. QRT-PCR analyses were performed using a Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, United States) and a Mx3005P qPCR System (Agilent Technologies Inc., Waldbronn, Germany). Efficiencies of the PCRs were calculated with slopes of standard curves of twofold dilutions. For each species two stable reference genes were used for normalization (Vandesompele et al., 2002; Schmidt and Delaney, 2010; Liu et al., 2012). The first sample of each experiment was used as a calibrator, which was set to one, and further samples are given as relative expression levels to the calibrator. For each condition three biological replicates with two technical replicates each were tested. A list of the primers used for each species can be found in Supplementary Table 1.

Enzyme Assay for CWINV, Soluble Acid Invertase, and Neutral Invertase

Proteins were extracted from 25 mg nectary tissue each as described by Wright et al. (1998). CW-INV activity was assayed according to Heineke et al. (1992). An aliquot of the protein extracts was added to 0.6 M sucrose and 0.125 M sodium acetate, pH 5.0. Soluble acid invertase activity was measured with the soluble protein fraction. An aliquot of the protein extracts was added to 0.6 M sucrose and 0.125 M sodium acetate, pH 5.0. Soluble neutral invertase activity was measured with the soluble protein fraction, too. An aliquot of the protein extracts was added to 0.6 M sucrose and 0.125 M sodium acetate, pH 7.5. After 10 min, the reaction was completely stopped by boiling and subsequently, the amount of glucose released was determined by coupled optical enzyme assay. All enzyme assays were conducted from six biological replicates with two technical replicates each. About 5 μL of nectar were also used to assay invertase activity.

RESULTS

Sugar Concentrations in Nectar and Nectaries During the Light and Dark Period

The sugar content in both nectar and nectaries was primarily composed of glucose, fructose, and sucrose. Other sugars, including maltose, were not found in any of the samples. The total sugar concentration in nectar ranged from 1042 ± 86 to 3183 ± 186 mM, depending on the species and collection time (**Figure 2**). The day-flowering species (*N. africana* and *N. tabacum*) had the highest nectar sugar concentration during the day, which decreased continuously at night. In the case of night-flowering tobacco (*N. benthamiana*, *N. sylvestris*), the lowest sugar concentration in nectar was also found in the first half of the night period, but the concentration increased during the second half of the night period. Day- and night-flowering *N. attenuata* behaved like *N. benthamiana* (**Figure 2**).

By measuring the sugar content in the nectaries in micromole per gram fresh weight and the water content of the nectaries, it was also possible to determine the sugar concentration in the nectaries. The total sugar concentration in the nectaries of all species was lower than in the nectar, ranging from 72 ± 6 to 613 ± 34 mM (**Figure 2**). The mean sugar concentration in the nectar was approximately three to fivefold higher than in the nectaries of day flowering species, and approximately eight to 10-fold higher in night flowering species and in *N. attenuata*. In the day-flowering plants, the highest sugar concentration in nectaries occurred either in the middle or at the end of the light period (2 or 8 p.m.). The same phenomenon applied to the mixed-type *N. attenuata*. In both night-flowering plants, the sugar concentration in nectaries increased sharply in the middle of the night at 2 a.m.

The leaves of these tobacco species also contained primarily sucrose, glucose, and fructose. Independent of the flowering time, the sugar content in leaves was higher at the end of the light period than at the end of the dark period (Supplementary Figure 1). When compared to nectaries or nectar, leaves had a significantly lower sugar concentration (10–60 mM). These results were derived from the sugar content per gram fresh weight and the corresponding water content (78–94%; data not shown).

Nectar samples have been tested for microbial contamination. However, no contaminations with yeast or bacteria in the different *Nicotiana* species were found and therefore externally induced changes in the nectar sugar profile due to microbial activity can be excluded.

Sugar Composition in Nectar and Nectaries During the Light and Dark Period

While the ratios of the three sugars within a species remained relatively constant, even during different collection times, the sugar ratio between species varied greatly in some cases (**Figure 3**). This phenomenon was observed for both nectar and nectaries. In the nectar of *N. africana*, the percentage of sucrose ranged from 3–8%, depending on the time of day. Other species with a low sucrose-to-hexoses ratio in nectar were *N. attenuata* and *N. benthamiana,* for which the proportion of sucrose ranged from 6–9 and 10–13%, respectively. Higher proportions of sucrose were found in *N. tabacum* and *N. sylvestris* (16–23 and 42–49%). In general, glucose and fructose were found to occur in similar proportions within a species.

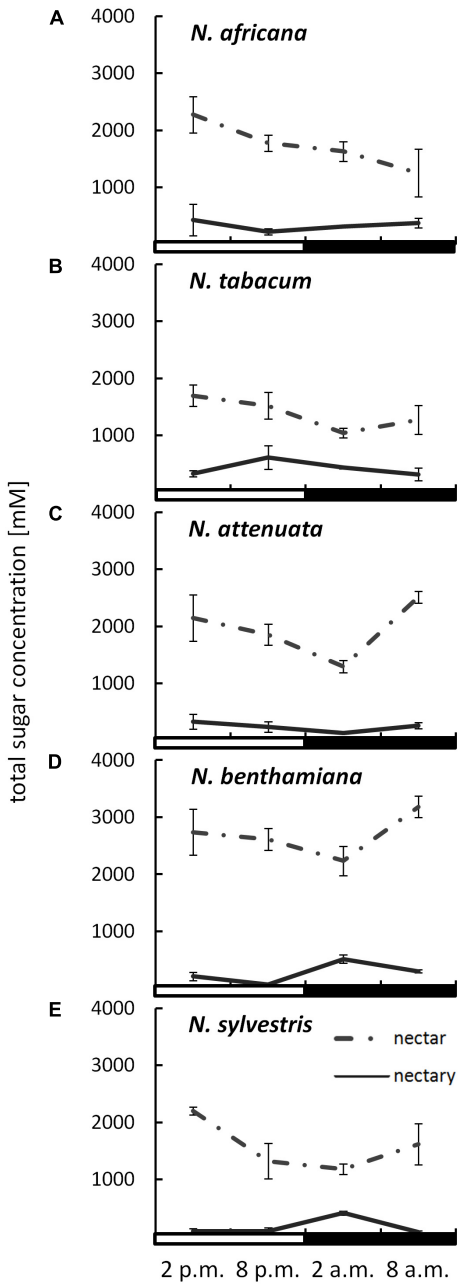

FIGURE 2 | Sugar concentrations in nectaries and nectar. Mean values from all measurements taken at one time point (n = 3) and the respective SDs were plotted; light and dark periods are indicated by white and black bars.
(A) *N. africana*, **(B)** *N. tabacum*, **(C)** *N. attenuata*, **(D)** *N. benthamiana*, and **(E)** *N. sylvestris*.

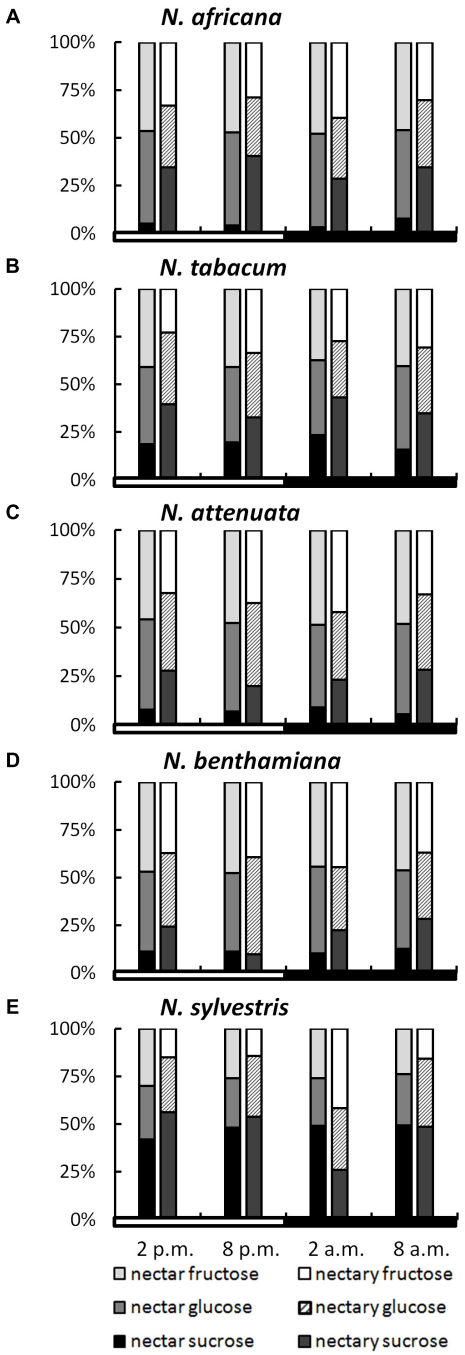

FIGURE 3 | Sugar percentages in nectaries and nectar. All percentages were calculated from mM; n = 3; light and dark periods are indicated by white and black bars; one pair of bars indicates one time point of sampling, with the Left bar representing nectar and the Right bar representing nectaries.
(A) *N. africana*, **(B)** *N. tabacum*, **(C)** *N. attenuata*, **(D)** *N. benthamiana*, and **(E)** *N. sylvestris*.

In nectaries, the distribution of sugars was also similar within a species during the light and dark period. In relation to nectar, the percentage of sucrose was higher in nectaries of all *Nicotiana* species, with the exception of *N. sylvestris* at 2 a.m. The percentage of sucrose was relatively low in *N. attenuata* and *N. benthamiana* (10–28%), medium in *N. africana* and *N. tabacum* (26–43%), and high in *N. sylvestris* (26–56%).

To assess whether a percentage increase of a given sugar in nectar was also reflected in the nectaries, the sugar content in both compartments was correlated. For glucose, no bivariate correlation was found (Pearson's r = 0.191,

TABLE 1 | Nectary- and Nectar-sugar-ratios (calc. from mM) Data were derived from **Figures 2**, **3**, the values of all measuring points were averaged.

Species	Nectary-sugar-ratio [S/(G+F)]	Nectar-sugar-ratio [S/(G+F)]	Difference between the ratios
N. africana	0.54	0.05	0.48
N. tabacum	0.61	0.24	0.37
N. attenuata	0.33	0.08	0.25
N. benthamiana	0.28	0.13	0.15
N. sylvestris	0.94	0.89	0.05

p = 0.420), whereas the percentage of both fructose and sucrose between the nectar and nectaries was correlated either highly significantly or significantly (fructose: Pearson's r = 0.574, p = 0.008**; sucrose: Pearson's r = 0.481, p = 0.032*).

However, in all species, the mean sucrose-to-hexoses ratio was higher in nectaries compared with nectar (**Table 1**). In general, the difference between the sucrose-to-hexoses ratios in nectaries and nectar was higher in light flowering species (Δ 0.37–Δ 0.48) compared with night flowering species (Δ 0.05–Δ 0.15).

Starch Content in Nectaries

The starch content measured in nectaries ranged from 0.9 ± 0.1 mg g^{-1} FW up to 20 ± 1.5 mg g^{-1} FW (measured as glucose equivalent; **Figure 4**). The values were significantly higher in night- than in day-flowering species (p = 0.025). The lowest starch contents during the light and dark period were found in the day-flowering species, as well as in *N. attenuata*. Moreover, in these species, the starch content was lower during the dark period and higher during the light period. In the night-flowering *Nicotiana* species, the highest starch contents were found both in the morning and in the evening (**Figure 4**). At 2 a.m., the night flowering species showed the lowest starch levels; thus, at the same time, the night-flowering species presented the highest sugar concentration. Because starch in plants is synthesized from glucose, it has been tested whether there is a correlation between the glucose and starch content in the nectaries, but no significant correlation was found between glucose and starch content or between fructose or sucrose and starch content.

Starch content in leaves ranged from 0.5 ± 0.1 to 40 ± 4.8 mg g^{-1} FW (measured as glucose equivalent; Supplementary Figure 2). For all five species, the starch content in the leaves was higher at the end of the light period compared with the end of the dark period (Supplementary Figure 2). The starch content of nectaries and leaves was not correlated.

Invertase Activity in Nectaries

Cell wall invertases in nectaries were active during the light as well as during the dark period. Measured activity ranged from 0.003 ± 0.001 to 0.059 ± 0.004 U mg^{-1} FW (**Figure 5**). Except for *N. africana*, the highest activity levels in all species were found at the middle of the light period, and then the activity decreased, regardless of when the plant opens its flowers. The activity of CW-INV in nectaries did not correlate with any of the sugars in the nectar or the nectaries.

The CW-INV activity in the leaves ranged from 0.003 ± 0.001 to 0.033 ± 0.004 U mg^{-1} FW (Supplementary Figure 3). Therefore, the activity levels were similar to those in the nectaries. CW-INV activity in the leaves fluctuated only slightly between the light and dark periods.

Soluble acid invertases in nectaries were also active during the light as well as during the dark period, but the mean activity was about threefold lower when compared to the CW-INV activity. Measured activity ranged from 0.003 ± 0.001 to 0.013 ± 0.007 U mg^{-1} FW (Supplementary Figure 4A). In the day-flowering species and in *N. attenuata*, the highest activity levels were found at the middle of the light period, whereas in the night-flowering species the highest activity levels were found at the middle of the dark period.

The activity of the soluble neutral invertase in the nectaries of the different *Nicotiana* species was very low (Supplementary Figure 4B). Measured activity ranged from 0.001 to 0.007 U mg^{-1} FW and no significant differences between the species or the sampling points were found.

Expression Levels of CWINV

The expression level of CW-INVs in the nectaries of the five *Nicotiana* species was also measured. Therefore, the expressed sequence tag (EST) of the CW-INV of each species was cloned. Specific primers were designed and used for quantitative RT-PCR. In the day-flowering and hexose-rich species, *N. africana,* the relative expression of CW-INV was very stable throughout the light and dark periods. In the other *Nicotiana* species, the expression level was slightly higher during the light period compared to the dark period, regardless of flowing time or the percentage of hexoses in the nectar (**Figure 6**). In most *Nicotiana* species, the course of the expression level was consistent with the invertase activity, especially for *N. tabacum* and *N. sylvestris* (**Figures 6B,E**), but less for *N. benthamiana* (**Figure 6D**). A comparison of the invertase expression level with the nectary sugar concentration revealed a non-homogeneous pattern: In *N. attenuata,* nectary sugars correlated strongly but not significantly with expression level (glucose: Pearson's r = 0.913, p = 0.458; fructose: Pearson's r = 0.917, p = 0.456; sucrose: Pearson's r = 0.917, p = 0.455), whereas in *N. benthamiana,* this correlation was strongly negative (glucose: Pearson's r = −0.887, p = 0.469; fructose: Pearson's r = −0.822, p = 0.497; sucrose: Pearson's r = −0.963, p = 0.437). For the remaining species, the correlation was generally lower.

Post-secretional Nectar Changes

To test for changes of the nectar sugar composition after secretion, nectar of all species was measured immediately after sampling, as well as 12, 24, and 48 h later. The results showed that the sugar concentrations were not changed significantly during

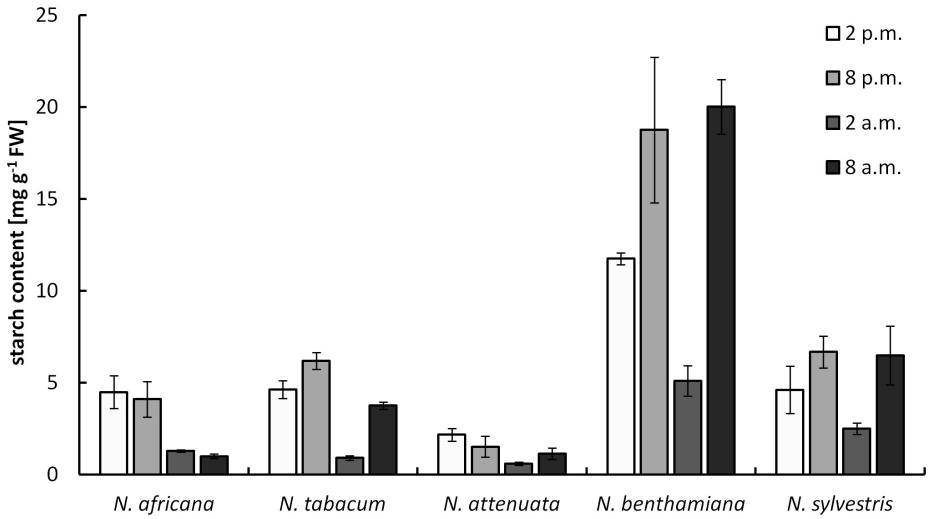

FIGURE 4 | Starch concentration in nectaries. Mean values from all measurements taken at one time point (n = 3) and the respective SDs were plotted.

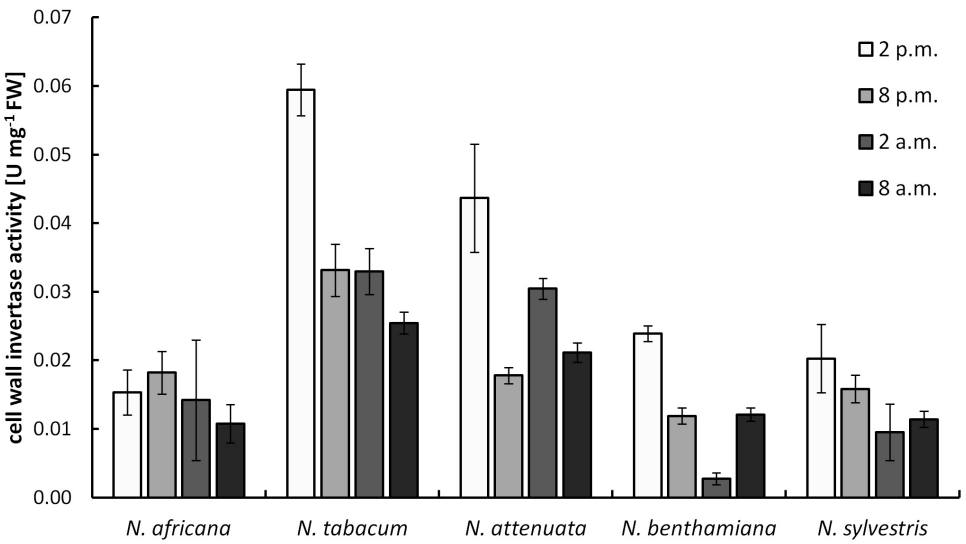

FIGURE 5 | Cell wall invertase (CW-INV) activity in nectary tissue. Mean values from all measurements taken at one time point (n = 3) and the respective SD were plotted.

this period (**Figure 7**). Minor fluctuations were likely caused by the high dilution factor (1: 2000) used to measure nectar with the HPLC. No invertase activity was found in any nectar sample.

DISCUSSION

Floral nectar is synthesized and secreted by different types of floral nectaries. Nectar composition varies between species, possibly to reward different types of pollinators. Until now, the plant-specific differences in nectar production and nectar secretion that lead to different nectar composition have not been fully understood.

Pre-secretory Modifications of Nectar Sugars

The phloem supplies the nectaries with sucrose (Lohaus and Schwerdtfeger, 2014). In contrast to phloem sap, where no hexoses are found (Knop et al., 2001; Nadwodnik and Lohaus, 2008), the nectar of the *Nicotiana* species contains substantial amounts of glucose and fructose, in addition to sucrose. Differences in the composition of nectar and phloem may be due to either metabolic processes in the nectaries during nectar secretion or post-secretional modification. To clarify this question, the sugar composition of the nectar and nectaries was compared.

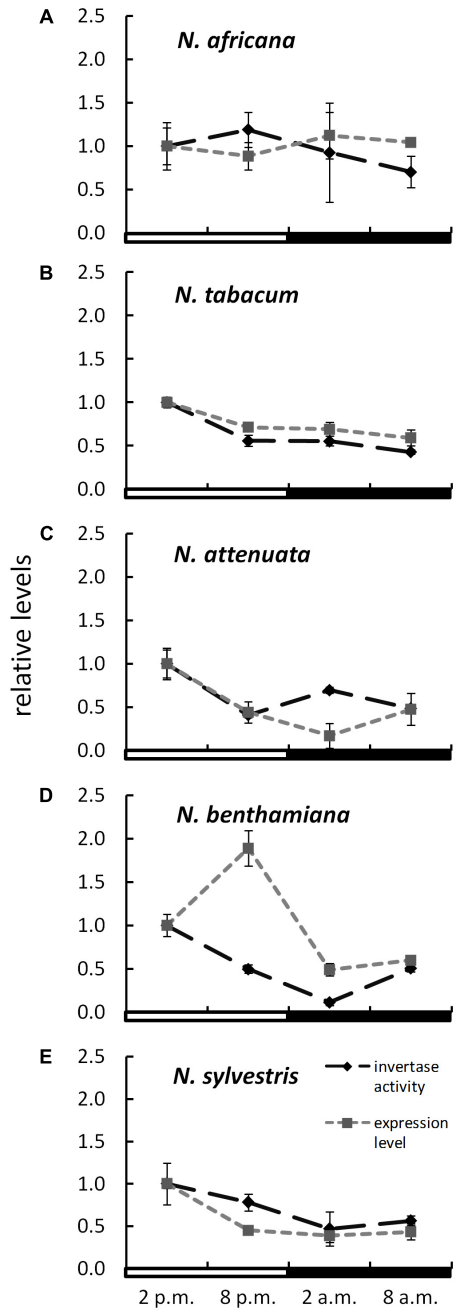

FIGURE 6 | Invertase activity in comparison to expression level in the nectaries of different *Nicotiana* species Activity and expression levels are given relative to the calibrator (2 p.m.). Mean values from all measurements taken at one time point (n = 6 for enzyme activity and n = 3 for expression levels) and the respective SD were plotted; light and dark periods are indicated by white and black bars. **(A)** *N. africana*, **(B)** *N. tabacum*, **(C)** *N. attenuata*, **(D)** *N. benthamiana*, and **(E)** *N. sylvestris*.

In the case of night-flowering tobacco, the lowest sugar concentration in nectar was observed in the first half of the dark period (**Figure 2**). This could be due to the fact that the nectar volume in these species is highest at this time (data not shown), and, therefore, the high water content ensures dilution. However, for day-flowering tobacco, the sugar concentration was also found to be lower during the dark period compared with the light period, even though the highest nectar volume is during the day, which contradicts the previous assumption. At night, phloem transport is reduced to approximately 40% of the daily rate (Riens et al., 1994), which means that less sucrose should arrive to the nectaries in darkness, and this could also be a reason for the observed fluctuations in the nectar sugar concentration. Therefore, it is generally easier for day-flowering plants to supply their nectar with nutrients for their pollinators, because they can process their metabolites directly from the phloem sap; in contrast, night-flowering plants, at least partially, have to store the metabolites (**Figure 8**). This finding corresponds to the differences in the starch content observed in the nectaries of day and night flowering species. In general, the night-flowering species had a higher starch content in the nectaries compared with the day-flowering species (**Figure 4**). Moreover, in night flowering species, the starch content decreased during the first half of the dark period, the time with high nectar production.

Starch accumulation may function as a form of sugar storage before anthesis (Weber et al., 1998), and starch degradation has been observed to occur before flower opening to provide additional sugar (Nepi et al., 1996; Horner et al., 2007; Ren et al., 2007b). In potato tubers (*Solanum tuberosum*), starch breakdown is triggered by decreased sucrose content (Hajirezaei et al., 2003). In this study, no overall correlation between starch and sucrose or hexoses in nectaries was observed, but in the case of nocturnal species, where there is a severe decrease of starch in the middle of the night, the sugar concentration was found to be significantly increased. Apart from that, there was no correlation between the starch content of the nectaries and leaves, thus the starch metabolism in the nectaries appears to function independently from the light-dependent starch metabolism of the plant.

Total nectary sugar concentration is highest at the time of flower opening, so sugar is likely provided for nectar production (**Figure 2**). There is a high correlation between the proportion of fructose and sucrose in nectaries and nectar. This suggests that the nectar sugar composition is already partly determined by the nectaries and is only partially adjusted during secretion. For glucose, this correlation is much lower. This phenomenon may be explained by the fact that some of the glucose is converted into starch and stored in the nectaries until it is used (Ren et al., 2007b).

Modulation During Nectar Secretion

The sugar concentration in nectar was three to 10-fold higher than in the whole nectarial cells. An increase in concentration due to evaporation can be neglected because the analyzed species have very long and narrow flower tubes, which protect the nectar from evaporation (Plowright, 1987; Tiedge and Lohaus, 2017). This suggests that active sugar transport is involved in nectar secretion, perhaps through monosaccharide transporters (MSTs) and/or sucrose transporters (SUTs). A monosaccharide/proton symporter (AtSTP1), which

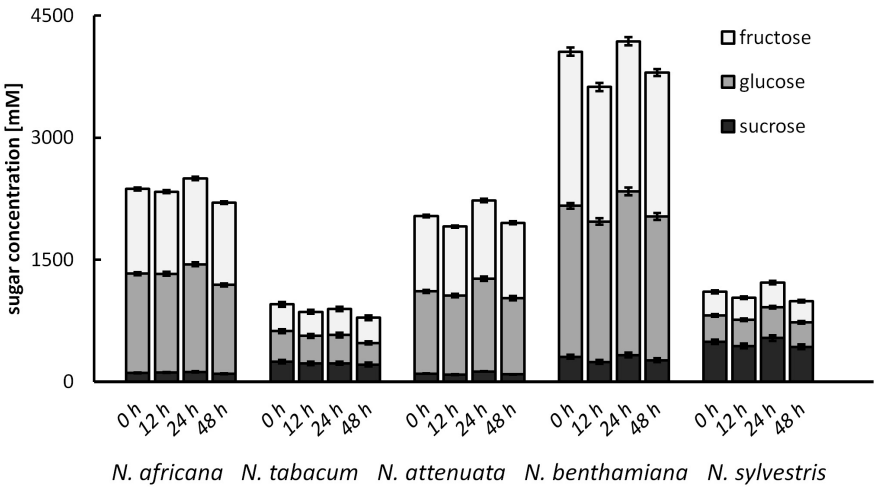

FIGURE 7 | Post-secretional changes in sugar content in the nectar All samples were analyzed immediately after collection, as well as 12, 24, and 48 h after collection; $n = 3$.

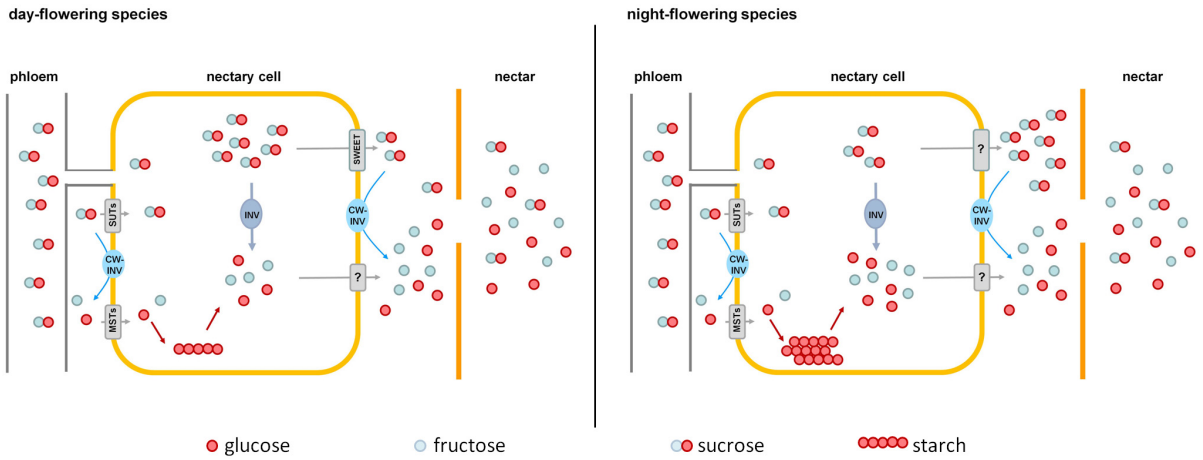

FIGURE 8 | Comparison of nectar sugar secretion in day- and night-flowering tobacco. Day-flowering species **(Left)** store less starch in their nectaries and contain more sucrose in the cytoplasm, which could be exported by SWEET-proteins along the concentration gradient. Night-flowering species store more starch in their nectaries and sucrose probably cannot be exported by SWEETs, since the concentration of sucrose in the cytoplasm is lower than in the nectar. In night-flowering species, sucrose cleavage within the nectaries accounts for a higher proportion of the hexose provision than cleavage during secretion, which is the opposite in day-flowering species. MSTs, monosaccharide transporters; SUTs, sucrose transporters; CW-INV, cell wall invertases; INV, invertase; SWEETs, sucrose efflux transporters; ?, unknown processes.

only transports glucose but not fructose, has been found in *Arabidopsis* flowers (Sherson et al., 2003). SUTs have already been found in tobacco, as well, e.g., NtSUT3 in tobacco pollen (Lemoine et al., 1999), but, so far, their occurrence and function in flowers and nectaries is not completely understood.

A class of transporters that are clearly involved in nectar secretion are so-called SWEET sucrose transporters. In *Arabidopsis* and *Nicotiana,* SWEET9 functions as a facilitated diffusion transporter for sucrose (Lin et al., 2014), and there is evidence that this transporter is more responsible for sucrose efflux from nectarial cells than for sucrose uptake. As previously mentioned, the sugar concentration in nectar relative to nectary cells was three to fivefold higher in diurnal species and eight to 10-fold higher in nocturnal species. Unfortunately, until now, nothing has been reported about the subcellular distribution of sugars in the parenchyma cells of nectaries. Assuming that the subcellular distribution of sucrose in nectarial cells is similar to the distribution in leaves (up to 50% sucrose in the cytosol; Nadwodnik and Lohaus, 2008) and the cytosolic compartment comprises about 20% of the nectarial cells (Wist and Davis, 2006; Gaffal et al., 2007), the sucrose concentration in nectarial cells can be extrapolated (**Figures 2**, **3**). In day-flowering species, the maximal sucrose concentration in the cytosol of nectarial cells was approximately 300–400 mM, and the corresponding concentration in nectar was approximately 100–300 mM. Similar

results were obtained for *N. attenuata*. Therefore, it is possible that facilitated diffusion transporters for sucrose mediate sucrose efflux from nectarial cells (**Figure 8**). In night-flowering species, the calculated concentration of sucrose in the cytosol of nectarial cells was approximately 200 mM, whereas the corresponding concentration in nectar was approximately 300–700 mM. In the latter case, facilitated diffusion of sucrose from the nectarial cells into the nectar is not possible. However, this does not exclude the possibility that different cell types in the nectaries contain different sugar concentrations and that facilitated diffusion of sugars occurs only in certain nectarial cells, whereas in other cells active sugar secretion may occur. This finding is in line with findings in *Arabidopsis*, where SWEET9 was localized at the basal part of the nectaries (Lin et al., 2014), and the conclusions drawn from other research in this area, which propose a division of nectary parenchyma into functional sub-domains (Roy et al., 2017).

Besides sugar transporters, invertases also appear to be part of the nectar metabolism. For this work, the expression of the CW-INV was investigated exclusively in nectary tissue. Not much is known about the regulation of CW-INV expression in nectaries, but this enzyme has already been studied in other plant organs. Invertase expression is regulated by multiple factors, for example, by carbohydrates (Koch, 1996), phytohormones (Wu et al., 1993), biotic and abiotic stress-related stimuli (Roitsch et al., 2003), and proteinaceous inhibitors (Krausgrill et al., 1996). So far, it has rarely been examined how nectar-related invertase expression in nectaries is regulated. The invertase found in *N. attenuata* is highly upregulated in parts of early corollas, such as nectaries, ovaries and anthers. When the flowering continues to ripen, the invertase expression decreases (*Na*DH; Brockmöller et al., 2017). Most nectar is produced during early flowering, while older flowers sometimes have no nectar at all. This fact also suggests that invertase plays a role in the production of nectar. For other *Nicotiana* species, no organ-specific expression data about CW-INVs are available yet. Sturm and Chrispeels (1990) found that carrot cell suspension cultures grown on either glucose, fructose, or sucrose have similar β-fructofuranosidase mRNA content, with slightly higher levels of mRNA in cells grown on glucose (Roitsch et al., 1995). In contrast, the expression of different β-fructofuranosidase genes can be repressed by glucose (Kunst et al., 1974; Sarokin and Carlson, 1984; Martin et al., 1987). For tobacco, this phenomenon may only be applicable for *N. benthamiana*, where there is a strong negative correlation between the nectar sugars in general and invertase expression levels. Furthermore, high expression levels resulting in high CW-INV activity would have been expected. This seems to be true especially for species with high sucrose content in nectar (*N. sylvestris* and *N. tabacum*). Nevertheless, post-transcriptional processes seem to be taking place, which prevent the entire transcript from being converted into active protein.

The activity of CW-INV in the nectaries of different *Nicotiana* species (0.003–0.06 U mg^{-1} FW; **Figure 5**) was similar to the activity of CW-INV measured in other hexose-rich tissues of different plant species (Weschke et al., 2003). Moreover, an increased invertase activity would be expected in plants with a high hexose concentration in the nectar (Ruhlmann et al., 2010). However, for *tobacco,* this assumption is not confirmed by the data, regardless of whether the species is hexose-rich or not. The same applies to changes in the vacuolar invertase activities (Supplementary Figure 4A). In day-flowering species the vacuolar invertase activity was slightly higher in the light period and in night-flowering species in the dark period, regardless of whether the species is hexose-rich or not. Furthermore, due to the low activity, the neutral invertase seems to have only a relatively small influence on the hexose production. There might be other mechanisms that play a role in the sugar composition, for example, the *in planta* regulation of the sucrose cleavage enzymes. In addition to sucrose cleaving enzymes, sugar synthesis enzymes could also be involved in nectar production. It has been shown that sucrose phosphate synthase is highly expressed in some nectaries and that its expression can be essential for nectar production (Lin et al., 2014).

For all five *Nicotiana* species, the sucrose proportion of the total sugar concentration was always lower in the nectar compared with the nectaries (**Figure 3**), perhaps due to the extracellular hydrolysis of sucrose by CW-INVs. Differences in the sucrose-to-hexose-ratio between the nectaries and nectar were more pronounced in diurnal species compared with nocturnal species (Δ 0.37–0.48 vs. Δ 0.05–0.15). Therefore, the cleavage of sucrose during secretion must be stronger in diurnal species (**Figure 8**). Due to the differences in sugar composition between nectaries and nectar, especially in day-flowering species, it can be assumed that the sugar composition is at least partly modified during secretion, either by the selective transport of sugars and/or the activities of sugar cleavage enzymes, like CW-INVs.

Post-secretory Modifications

No changes in nectar sugar concentration were observed after secretion in the tobacco species analyzed in this study and no invertase activity was detectable in nectar. In acacia, a significant post-secretional modification of extrafloral nectar by invertase has been demonstrated (Heil et al., 2005). Invertase activity in the nectar was also measured in *Cucurbita pepo*, but it was too low to significantly change the sugar profile (Nepi et al., 2012). Although other sugar-cleaving enzymes, such as glucosidase, have been identified in the nectar of *N. attenuata*, no invertases have been found in the tobacco nectar so far (Seo et al., 2013). This means that the nectar sugar composition must be already determined during the final stage of secretion, rather than undergoing post-secretory modification.

CONCLUSION

Nectar sugar composition must be determined by metabolic processes in nectaries as well as during secretion (**Figure 8**). Sucrose is transported to the nectaries via the phloem. Within the nectaries, sucrose is hydrolyzed into hexoses, and a portion of the sugars is transiently stored as starch until anthesis, especially in night-flowering species. At anthesis, starch is

converted into sucrose and hexoses. Sugars are exported out of the nectarial cells, likely by facilitated diffusion transporters (day-flowering species) or active transporters (night-flowering species). In the nectary tissue as well as during nectar secretion, some of the sucrose is hydrolyzed into glucose and fructose by the activity of CW-INVs, which explains the higher proportion of hexoses in nectar in comparison to nectaries. Sucrose cleavage is likely higher pre-secretional in night-flowering (possibly by vacuolar invertases) and during secretion in day-flowering species. Furthermore, post-secretional modification of the sugar composition in nectar is not probable. However, CW-INV alone cannot be responsible for the differences in hexoses concentration, and, therefore, other enzymes seem to play important roles in determining the nectar sugar composition.

AUTHOR CONTRIBUTIONS

KT and GL contributed conception and design of the study and wrote the manuscript. KT performed the statistical analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00622/full#supplementary-material

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Nectar Analysis Throughout the Genus *Nicotiana* Suggests Conserved Mechanisms of Nectar Production and Biochemical Action

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We have evaluated the floral nectars of nine species from different sections of the genus *Nicotiana*. These nine species effectively cover the genus. We found that the nectary glands from these different species showed similar developmental regulation with swelling of nectaries during the first half of development and a distinct color change in the nectary gland as development approaches anthesis. When we examined the composition of the nectar from these nine different species we found that they were similar in content. Carbohydrate compositions of these various nectars varied between these species with *N. bonariensis* showing the highest and *N. sylvestris* lowest level of sugars. Based upon the amount of carbohydrates, the nectars fell into two groups. We found that hydrogen peroxide accumulated in the nectars of each of these species. While all species showed the presence of hydrogen peroxide in nectar, the quantitative amounts of hydrogen peroxide which was very high in *N. rustica* and *N. bonariensis,* suggesting be a common characteristic in short flower *Nicotiana* species. We further found that the antioxidant ascorbate accumulated in nectar and β-carotene accumulated in nectaries. β-carotene was most high in nectaries of *N. bonariensis*. We also examined the presence of proteins in the nectars of these species. The protein profile and quantities varied significantly between species, although all species have showed the presence of proteins in their nectars. We performed a limited proteomic analysis of several proteins from these nectars and determined that each of the five abundant proteins examined were identified as Nectarin 1, Nectarin 3, or Nectarin 5. Thus, based upon the results found in numerous species across the genus *Nicotiana*, we conclude that the mechanisms identified are similar to those mechanisms found in previous studies on ornamental tobacco nectars. Further, these similarities are remarkably conserved, throughout the genus *Nicotiana*.

Keywords: *Nicotiana*, floral nectar, nectaries, carbohydrate in nectar, nectary carotenoids, hydrogen peroxide, proteins in nectar

Abbreviations: EST, expressed sequence tagged mRNA; Floral Nectary stages: S6, Stage 6 (immature; beginning of metabolic switch); S9, Stage 9 (immature; pre-secretory, S12, Stage 12 (mature; anthesis); PF, post-fertilization; LxS8, an interspecific cross of *Nicotiana langsdorffii* × *N. sanderae* long studied for nectar/nectary research.

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INTRODUCTION

The floral nectary is a unique organ that undergoes a complex developmental pathway. Over the past two decades, we have investigated the biochemistry of floral nectar and the floral nectary gland. These studies have focused on an interspecific cross of *Nicotiana langsdorffii* × *N. sanderae* (LxS8). This cross has a number of advantages that permit the biochemical analysis of these tissues. First, plants of this cross have very large nectary glands and produce copious quantities of floral nectar. This has permitted large-scale biochemical analyses of both floral nectar and the floral nectary gland. These studies have shown the floral nectar contains a limited array of proteins termed Nectarins (Carter et al., 1999; Carter and Thornburg, 2004a; Naqvi et al., 2005; Park and Thornburg, 2009). These nectar proteins function together in a novel biochemistry pathway termed the Nectar Redox Cycle. The nectar redox cycle is an oxidative cycle that produces very high levels of hydrogen peroxide as a defense compound (Carter and Thornburg, 2004a).

In nectaries of *Nicotiana*, this oxidative process is initiated by a NADPH oxidase (Carter et al., 2007) that produces high levels of superoxide (Thornburg et al., 2003) and subsequently, the Nectarin 1 superoxide dismutase in Nectar Redox Cycle pathway converts the superoxide into high levels of hydrogen peroxide, up to 4 mM, in nectar (Carter and Thornburg, 2000), that is toxic to multiple microorganisms (Carter et al., 2007). These levels of hydrogen peroxide serve to protect flowers from microbial infections (Thornburg et al., 2003).

In addition, we have also characterized the biochemistry of the nectary gland during floral development. These studies have shown that the nectary glands accumulate very high levels of photosynthate that is stored as starch during the first 4–5 days of floral development which is termed the growth phase (Ren et al., 2007a). Subsequently, about floral stage 9, (about 24 h before anthesis) there is shift in metabolism from starch anabolism to starch catabolism (Ren et al., 2007a) that results in the release of high levels of free sugar that flows into the biosynthesis of antioxidants (ascorbate and β-carotene) (Ren et al., 2007b) and into nectar via the sugar transporter SWEET9 (Lin et al., 2014). This shift in metabolism is transcriptionally controlled by a novel floral transcription factor, MYB305 (Liu et al., 2009). MYB305 is expressed about floral stage 6 (Liu et al., 2009), prior to the metabolic switch that leads to starch breakdown and sugar production. Of note, knockdown of the MYB305 protein in floral nectaries results in plants with reduced production of antioxidants as well as reduced levels of sugar in floral nectar.

Accompanying this maturation process, the nectary morphs into the plant's premier secretory organ. The primary component of nectar secretions is a carbohydrate-rich material as a reward for pollinator activity. In *Nicotiana* plants the secretion of nectar begins about floral stage S10, and reaches a maximum at floral stage S12. There are three main carbohydrates that make up the nectar of most species (Bolten et al., 1979). The carbohydrates produced not only enter the secretion pathway to form nectar, but carbohydrates such as glucose, can also act as precursors for the biosynthesis of important nectar/nectary compounds such as ascorbate, oxalate and β-*carotene* that are crucial in redox metabolism (Horner et al., 2007).

In addition to compensating pollinators for visiting, tobacco nectar also shows defensive properties (Thornburg et al., 2003) while some of these defenses are related to redox activity (Carter and Thornburg, 2004b,c). There are also proteinaceous defenses in the genus *Nicotiana* (Carter and Thornburg, 2000, Carter and Thornburg, 2004a). The major nectar protein (*NEC1*) begins to be expressed about floral Stage 10 (Carter et al., 1999) and nectar secretion itself begins prior to Stage 11 (Ren et al., 2007b). Nectar from the interspecific cross produces a limited array of proteins that function together to a developmental NADPH oxidase is expressed initiating the Nectar Redox Cycle (Carter and Thornburg, 2004a) just before anthesis at Stage 12 (Carter et al., 2007).

To extend these observations, we have also examined the nectar biochemistry from *Petunia* sp., a close relative of tobacco. Those studies demonstrated that the nectar biochemistry of petunia is significantly different that or ornamental tobacco. First, petunia does not produce the high levels of hydrogen peroxide that are found in tobacco nectars and second, the nectar proteins found in petunia nectar are very different from those produced in tobacco nectars (Hillwig et al., 2010b). Because petunia and tobacco nectars are so very different, we initiated the current work is evaluate nectar biochemistry throughout the genus *Nicotiana*. We therefore have chosen a number of *Nicotiana* species that broadly represent the breadth of the *Nicotiana* to characterize their nectar production.

MATERIALS AND METHODS

Materials

The materials used in these studies were obtained from either Fisher Chemical Co[1]. or Sigma Chemical Co[2]. and were of the highest quality available.

Plant Materials

The tobacco species used for this study are shown in **Table 3**. Seeds were obtained from the United States Department of Agriculture National Genetic Resources Program[3], plants were grown from seed in the greenhouse and when approximately 15 cm tall, these plants were transplanted to individual 30 cm pots containing a local potting mix. Plants were grown under 16 h day/8 h night conditions until flowering. Flowers were staged as described in (Koltunow et al., 1990).

Floral Anatomy

To evaluate the floral morphology of these different *Nicotiana* sp., we characterized the size of the floral opening, where insects enter, the floral size, depth of the floral tube. Analysis of each

[1]www.fishersci.com

[2]www.sigmaaldrich.com

[3]http://www.ars-grin.gov

of these features was characterized from at least 10 flowers each from three different tobacco plants.

Floral size was measured using a digital micrometer, placing one tine at the base of the flower and the other at the corolla-floral tube junction. Likewise, the floral opening was also measured using a digital micrometer, placing both tines at opposite faces of the corolla's opening. The depth of the floral corolla was measured by inserting a short length of monofilament fishing line (30# test) until it stopped at the base of the gynoecium. The corolla-floral tube junction was then marked on the line, and after removing the line the depth of the corolla was measured.

Carbohydrate Analyses

Nectar was collected from flowers, in the first hours of the day, as previously described (Carter et al., 1999; Naqvi et al., 2005). For quantitative analyses, 100 μL of raw nectar was collected and diluted (1:1000) using double distillated water. The samples were immediately returned to the laboratory for carbohydrate quantification. The levels of sucrose, glucose, and fructose were evaluated using the sucrose/D-glucose/D-fructose determination kit (Boehringer Mannheim/r-Biopharm[4], catalog no. 10716260035), according to the manufacturer's directions.

Protein Quantification

Protein concentrations were determined by the dye-binding method described by (Bradford, 1976), with bovine serum albumin (BSA) as the standard.

SDS-PAGE

Protein profiles from raw nectar were analyzed by SDS-PAGE according method described by (Laemmli, 1970).

Hydrogen Peroxide in Nectar

Hydrogen peroxide in nectar was evaluated using the FOX reagent according by (Bleau et al., 1998; Hillwig et al., 2010b). Twenty-five microliters of nectar was added to 975 μL of distilled water. For analysis of H_2O_2 200 μL of diluted nectar was used in the assay reaction with Fox reagent. The FOX reagent contained sulfuric acid 1.2 mM, xylenol orange 0.1 mM, ferrous ammonium sulfate 0.25 mM and sorbitol 0.1 mM. The H_2O_2 concentration in nectar was determined from a standard curve by measurement of the absorbance 560 nm.

β-Carotene Analysis

To evaluate the levels of β-carotene in nectaries, we isolated 40 mg of nectary tissue from stage 12 flowers of each species. Care was taken to insure that non-nectary tissue was excluded from these samples as described (Horner et al., 2007). Carotenoids were extracted from the homogenate using two 1 mL aliquots of acetone followed by a 1 mL aliquot of hexane. The organic layers were combined, dehydrated with anhydrous sodium sulfate, evaporated to dryness, and taken up in 50-μL hexane for analysis. Carotenoid levels were estimated by absorption at 450 nm. The β-carotene was confirmed within each species by thin layer chromatography (TLC) on silica gel plates using an acetone: hexane (9:1) mobile solvent as previously described (Horner et al., 2007).

Ascorbic Acid Analysis

The ascorbic acid analysis was performed according method described by Horner et al. (2007). For these analyses, either nectar was harvested from stage 12 flowers of each species. For analysis of raw nectar, 50 μL of nectar was added to 150 μL of distilled water. An aliquot of 50 μL was used for assay in a total volume of 2 ml of 1% oxalic acid. This was titrated to a pink endpoint with 0.05% 2,6-dichlorophenolindophenol (DCIP) in 0.1 M phosphate buffer, pH 7.0. A standard curve using ascorbate 0–20 μg of ascorbate was prepared to quantitate levels of ascorbate.

Mass Spectrometry (LC-MS/MS)

The proteomic analysis was performed to identify proteins in nectars at the Iowa State University protein facility[5]. The nectar of different species was initially analyzed by SDS-PAGE according method described previously (Laemmli, 1970). Afterwards, the selected bands were excised and the pieces transferred to a 0.6 mL methanol, washed and then added 20 μl of 1% acetic acid. Next, the proteins were digested in solution with trypsin/Lys-C (Promega). The peptides were then separated by liquid chromatography and analyzed by MS/MS by fragmenting each peptide on Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer from Thermo Scientific. Raw data were analyzed using Thermo Scientific's Proteome Discoverer Software and the data searched using publically available databases. Bovine serum albumin was used as an internal calibration standard.

Statistical Analysis

To perform the biochemical analysis we used three different tobacco plants and the floral nectar or nectaries collected from multiple flowers of each single plant to compose three independent biological replicates. The one way ANOVA test was performed to determine if there is a significant difference between mean of the each specie from the total and Tukey's test, at $p < 0.05$ significance level, was used to analyze the differences between species. The statistical analysis was performed using the NCSS statistical software[6].

RESULTS

From earlier studies, we observed that the nectar of ornamental tobacco differed significantly from the nectar of the closely related petunia (Hillwig et al., 2010a). To investigate this observation, we decided to examine the nectars from a variety of tobacco species across the genus *Nicotiana* to determine whether differences were observed within the tobacco genus. Because our earlier work was done with a species cross that fell within the section Alatae. Then based upon the phylogenetic studies of Bogani et al. (1997), Chase et al. (2003), and Clarkson et al. (2004) we

[4]www.r-biopharm.com

[5]www.protein.iastate.edu

[6]www.ncss.com

selected five additional *Nicotiana* sections to enhance diversity within the genus *Nicotiana*. We obtained seed from numerous species of these sections from the United States Department of Agriculture National Genetic Resources Program[7]. These were grown to maturity and based upon growth characteristics as well as previously published analyses of pollinator preferences, we chose the *Nicotiana* species shown in **Table 1** for these analyses.

Once the selected plant species were growing and flowering, we compared the floral characteristics of these species. For these values, we measured floral opening (throat width), the corolla length (floral base to corolla), and the depth of the floral corolla. Our interests were to determine the size of a pollinating insect that could enter the floral opening, as well as the depth of the corolla to determine the minimum length of the pollinator's proboscis. This analysis shown in **Table 2** illustrate that there are different categories of flower size among our selected group. Long flowers (>7 cm) include the *N. alata* and *N. sylvestris* ($q_s = 1.53$, $p < 0.899$). The shortest flowers (<2.5 cm) include *N. rustica* ($q_s = 33.22$, $p < 0.01$) and *N. bonariensis* ($q_s = 34.22$, $p < 0.01$). The intermediate sized flowers ranged from (2.5 cm to 5 cm) encompass the remainder *N. benthamiana* ($q_s = 19.42$, $p < 0.01$), *N. plumbaginifolia* ($q_s = 20.44$, $p < 0.01$), *N. glauca* ($q_s = 22.49$, $p < 0.01$), *N. clevelandii* ($q_s = 25.55$, $p < 0.01$), and *N. langsdorffii* ($q_s = 29.22$, $p < 0.01$). We also determined the depth of the corolla and we found that the ratio of the corolla depth to the flower size was different, from 68% in *N. glauca* to 90% in *N. alata*. Thus, we found that the length of the floral tube is indicative of the length of the pollinator's proboscis required to reach any nectar at the base of the flower. Also shown in **Table 1** is the pollinator syndrome that is used by these species. In species with long flowers (*N. alata* and *N. sylvestris*) are preferred by hawkmoth, while species with intermediate or short size flowers (*N. glauca* or *N. langsdorffii*) are preferred by bird, hummingbird or bee.

Carbohydrate in Nectar

To begin analysis of nectar from these species, we examined the nectar carbohydrate from each of the selected plant species. Using a Sucrose/Glucose/Fructose analysis kit from Boehringer Mannheim/r-Biopharm, we measured each of these components and determined the molar ratios of each of these sugars in the different nectars (**Table 3**). These carbohydrate composition data (**Figure 1**) show two different groups: Group 1 – *N. glauca, N. benthamiana, N. clevelandii, N. alata, N. sylvestris*, *N. rustica,* and *N. plumbaginifolia* composed mainly of night flowering *Nicotiana* species showed the lower levels of sugars (<560 mM), while that day flowering *Nicotiana* species *N. bonariensis* and *N. langsdorffii,* showed the higher levels of sugars content (>1000 mM). For most of these species such as, *N. benthamiana*, *N. clevelandii*, *N. sylvestris,* and *N. plumbaginifolia*, the molar ratio of Glucose to Fructose was very similar. However, for a few species, notably the day flowering *Nicotiana* species, *N. glauca* and *N. rustica*, there was significantly more Fructose than Glucose. Similar observations have been made for these species (Tiedge and Lohaus, 2017), and recent findings Tiedge and Lohaus (2017) suggest that the differences in nectar sugars composition may be implicated with different mechanisms of secretion between day/night flowering *Nicotiana* species.

Nectary Carotenoids

After analyzing the floral characteristics and the carbohydrate composition of the nectars, we then examined the gynoecium and nectary gland of each of these species. Because of our interest in the development of the floral nectary during the process of floral growth, we examined the gynoecia of these species at four different floral stages: Stage 6 (pre secretion), Stage 9 (at the time of the metabolic switch, (Ren et al., 2007b), Stage 12 (anthesis, with full nectar secretion) and the Post-fertilization Stage (48 h after pollination). These stages are shown in Supplementary Figures S1–S4. In all cases, the gynoecium and nectary gland from each species increase in size and the color changes from light yellow or lime green at the earliest stages to a bright orange in nectaries of mature stages. The observed changes were very similar to the development of the nectaries of the interspecific cross LxS8 (Horner et al., 2007). Based upon the obvious swelling of the nectaries and the noticeable color changes, we hypothesized that similar developmental pathways (involving carotenoid accumulation (Horner et al., 2007) and starch buildup and breakdown (Ren et al., 2007b) likely exist in these different *Nicotiana* species. One striking feature that we observed was extreme levels of carotenoids that were present in the nectaries of *N. bonariensis*. This is shown best by comparing the color of *N. bonariensis* (Supplementary Figure S3, #15 and #24) with similarly staged nectaries of the other species in Supplementary Figure S3.

[7] www.ars-grin.gov

TABLE 1 | *Nicotiana* species selected for these studies.

Number	Species	Section	Pollination syndrome	Reference
1	*N. rustica*	Paniculatae	Moth	Anon, 1972–2017a
2	*N. glauca*	Noctiflorae	Birds	Ollerton et al., 2012
3	*N. benthamiana*	Suaveolentes	Open, moth, bee, other	Anon, 1972–2017a
4	*N. clevelandii*	Polydicliae	Open, moth, bee, other	Anon, 1972–2017b
5	*N. sylvestris*	Petunoides	Hawkmoth	Mahr, 2013
6	*N. plumbaginifolia*	Alatae	Hawkmoth	Kaczorowski et al., 2005
7	*N. bonariensis*	Alatae	Small moth	Kaczorowski et al., 2005
8	*N. alata*	Alatae	Hawkmoth	Kaczorowski et al., 2005
9	*N. langsdorffii*	Alatae	Hummingbird, bee	Kaczorowski et al., 2005

TABLE 2 | Floral characteristics among the selected *Nicotiana* sp. dimensions were determined as outlined in the Section "Materials and Methods."

Species	Floral opening (mm)	Floral size (cm)	Corolla depth (cm)	Ratio (Cd/Fs) × 100%
N. rustica	5.1 ± 0.7^{c}	1.6 ± 0.1^{e}	1.3 ± 0.2^{d}	81%
N. glauca	4.9 ± 0.0^{b}	3.7 ± 0.1^{bc}	2.5 ± 0.1^{c}	68%
N. benthamiana	2.8 ± 0.3^{c}	4.3 ± 0.5^{b}	3.6 ± 0.5^{b}	84%
N. clevelandii	3.6 ± 0.5^{c}	3.1 ± 0.2^{cd}	2.2 ± 0.2^{c}	71%
N. sylvestris	2.9 ± 0.3^{c}	7.8 ± 0.6^{a}	6.9 ± 0.6^{a}	88%
N. plumbaginifolia	3.1 ± 0.3^{c}	4.1 ± 0.3^{b}	3.2 ± 0.2^{b}	78%
N. bonariensis	2.9 ± 0.2^{c}	1.4 ± 0.1^{e}	1.0 ± 0.1^{d}	71%
N. alata	7.9 ± 0.5^{a}	8.1 ± 0.5^{a}	7.3 ± 0.5^{a}	90%
N. langsdorffii	5.7 ± 0.5^{b}	2.4 ± 0.2^{de}	1.7 ± 0.1^{d}	71%

Different letters mean significant differences between species within the same characteristics at $p < 0.05$ for significance level.

TABLE 3 | Free sugars composition in nectar of different *Nicotiana* species.

Species	Sugar Concentration (mM)				Sugar (%)			Molar ratio			Ratio	
	S	*G*	*F*	Total	*S*	*G*	*F*	*S*	*G*	*F*	*F/G*	*S/(G+F)*
N. glauca	244 ± 2^{d}	7 ± 2^{h}	79 ± 2^{f}	330 ± 3^{g}	24 ± 1	1 ± 0	8 ± 3	0.7	0.0	0.4	11.0	1.8
N. benthamiana	235 ± 7^{d}	68 ± 3^{e}	78 ± 1^{f}	381 ± 1^{f}	23 ± 1	7 ± 1	8 ± 1	0.7	0.4	0.4	1.0	0.9
N. clevelandii	226 ± 5^{d}	88 ± 2^{d}	95 ± 2^{e}	409 ± 3^{e}	22 ± 3	9 ± 2	10 ± 1	0.7	0.5	0.5	1.0	0.7
N. sylvestris	148 ± 3^{f}	31 ± 7^{f}	40 ± 9^{h}	219 ± 5^{i}	15 ± 2	3 ± 1	4 ± 1	0.4	0.2	0.2	1.0	1.0
N. alata	194 ± 5^{e}	22 ± 1^{f}	63 ± 2^{g}	279 ± 3^{h}	19 ± 2	2 ± 1	6 ± 1	0.6	0.1	0.3	3.0	1.5
N. plumbaginifolia	286 ± 2^{c}	141 ± 2^{c}	150 ± 1^{d}	577 ± 2^{c}	28 ± 2	14 ± 1	15 ± 1	0.8	0.8	0.8	1.0	0.5
N. rustica	236 ± 4^{d}	31 ± 3^{f}	191 ± 3^{c}	458 ± 4^{d}	23 ± 1	3 ± 1	19 ± 1	0.7	0.1	1.1	10.0	0.6
N. langsdorffii	715 ± 1^{b}	195 ± 3^{b}	239 ± 7^{b}	1149 ± 1^{b}	71 ± 1	20 ± 1	24 ± 1	2.1	1.1	1.3	1.2	0.9
N. bonariensis	794 ± 4^{a}	282 ± 4^{a}	360 ± 1^{a}	1436 ± 1^{a}	79 ± 1	28 ± 1	36 ± 1	2.3	1.6	2.0	1.3	0.6

Different letters mean significant differences between species within the same characteristics at $p < 0.05$ for significance level.

To confirm our hypothesis that similar processes were occurring in the nectaries of these different species, we investigated the biochemistry of these different nectary glands. In LxS8, the orange color arises from β-carotene that is produced from isopentenyl pyrophosphate (IPP) arising from the dimethylallyl pyrophosphate (DMAPP) pathway (Horner et al., 2007). Therefore, we examined the level of β-carotene that was present in the Stage 12 floral nectaries of each species. As shown in **Figure 2A**, a bright orange pigment that co-chromatographed with β-carotene (R_f = 0.95) was present in the nectaries of each of the *Nicotiana* species. The amount of β-carotene varied significantly between *N. bonariensis* and other species showing the highest levels. Other species such as *N. sylvestris*, *N. benthamiana,* and *N. glauca* showed much lower levels of β-carotene. Note that several intermediate pigments that were also yellow were also observed. These were identified as lutein and xanthophyll by virtue of their R_fs = 0.80 and 0.17 (Schoefs, 2005). For each of the selected species, we further spectroscopically quantified the level of β-carotene. The results shown in **Figure 2B**, mirror the levels that were chromatographically identified in **Figure 2A**. In this analysis, *N. bonariensis* showed the highest levels of β-carotene ($p < 0.001$, df = 17, n = 18), confirming the observations of nectaries shown in the Supplementary Figure S3.

In addition to the presence of β-carotene in the nectary gland, the LxS8 interspecific cross also showed an additional antioxidant present in soluble nectar, ascorbate (Carter and Thornburg, 2004b). To determine whether these *Nicotiana* species also express this important nectar antioxidant, we evaluated whether ascorbate was present in the nectar of these selected species. The highest levels ascorbate was evidenced in *N. alata* and varied significantly ($p < 0.001$, df = 25, n = 27) of the other species, while, *N. langsdorffii* and *N. sylvestris* showed the lower levels, **Figure 6**.

Hydrogen Peroxide in Nectar

Previous analyses have demonstrated that LxS8 tobacco nectar had high levels of hydrogen peroxide (Carter et al., 1999). To determine whether other *Nicotiana* species also showed high levels of hydrogen peroxide, nectars were collected and their hydrogen peroxide content were measured with the FOX reagent method as described in Section "Materials and Methods." As shown in **Figure 3**, the nectar of all species do indeed contain hydrogen peroxide. However, two species, *N. rustica* and *N. bonariensis* had very high levels of hydrogen peroxide, 2.14 and 1.84 $\mu mol.ml^{-1}$, respectively. This could correlate with the high levels of sugars that were found in these species, especially in *N. bonariensis*. The high levels of sugar demonstrated in these species (**Table 3**) could increase the attractiveness of pollinators, having easier access to the nectar due to the floral characteristics (**Table 2**) increasing the colonization by microorganisms. The high levels of hydrogen peroxide would be a mechanism of control of microorganisms in nectar. In the other species, we

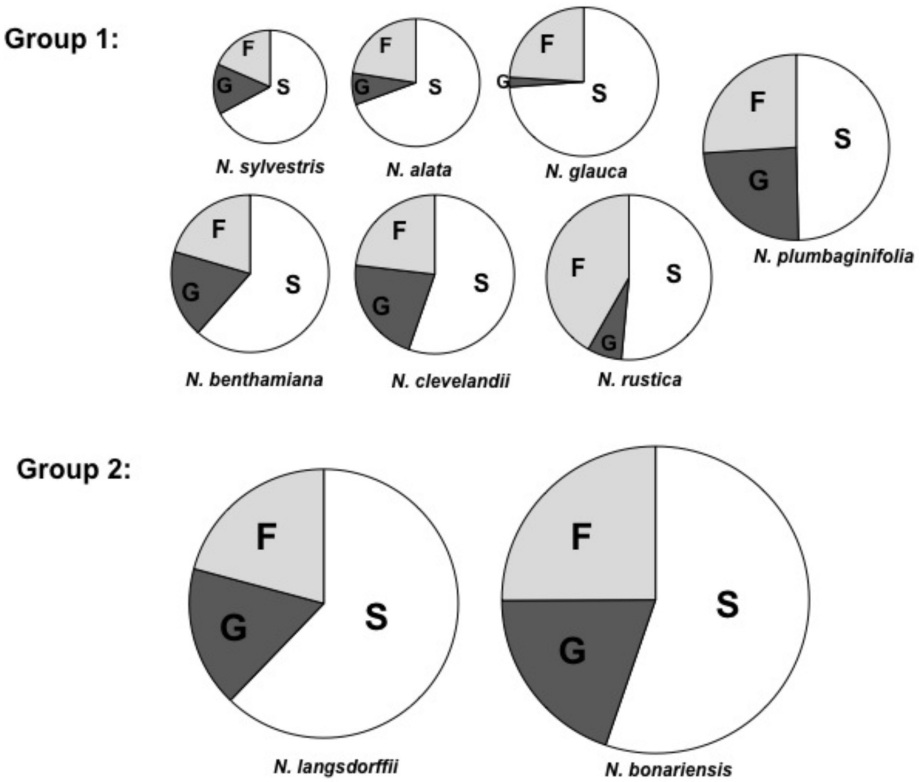

FIGURE 1 | Carbohydrate composition of selected tobacco species. Proportions of fructose (F), glucose (G), and sucrose (S) in nectar. The area of each circle represents the total mass of sugar found in each nectar sample. Each segment represents the mean of three samples. Averages ± standard deviation ($N = 3$). All characteristics of each nectar are present in **Table 3**. Group 1, total nectar carbohydrate < 560 mM, Group 2, total nectar carbohydrate > 1000 mM.

found that the level of hydrogen peroxide was lower. Reasons for this are unclear, but may be related by altered regulation between the species in these complex pathways.

Proteins in Nectar

To determine whether the different *Nicotiana* species also showed the presence of proteins in their nectars, we quantitated the amount of protein present in the nectars from each of these species. As shown in **Figure 4** the variability of nectar proteins was quite large, with some species such as *N. glauca* and *N. sylvestris* having very little protein in their nectars (0.044 μg protein/μL of nectar) while other species such as *N. clevelandii*, *N. rustica*, and *N. bonariensis* containing higher concentrations of protein in their nectars (up to 0.778 μg protein/μL of nectar).

Once we had confirmed that these species do indeed contain nectar proteins, we next wanted to identify the nectar proteins in these different species. First we investigated the profile of proteins in nectars among the species. SDS-PAGE analysis showed different profiles of the proteins in nectars **Figure 5** distributed between 70 and 20 kDa. The protein profile of *N. rustica*, *N. bonariensis*, *N alata,* and *N. langsdorffii* were very similar to the protein profile observed in the LxS8 interspecific cross (Carter and Thornburg, 2004a) and suggested that the nectarins found in LxS8 may also accumulate in nectar of these other *Nicotiana* species. The protein quantification also varied significantly ($p < 0.001$, df = 17, $n = 27$) among the species.

Based upon the SDS PAGE protein gels, it initially appears that there are many different proteins present in these different *Nicotiana* species. Therefore, we excised five of these proteins from the gel (identified by red spots) and following trypsin digestion; we subjected them to proteomic analysis. The results of this analysis is shown in **Table 4** and in detail in Supplementary Figures S5A–C. The spots 1 (from *N. benthamiana*) and 2 (from *N. clevelandii*) shown in **Table 4**, were identified as Nectarin 1-like superoxide dismutases although these proteins had showed differences between the predicted molecular mass by SDS-PAGE 28 and 23 kDa, and the mass found by mass spectrometry 24.6 kDa. The different molecular weight likely is due the degree of glycosylation of Nectarin 1 proteins as was showed previously by Carter et al. (1999).

Furthermore, the data suggest that Nectarin 1 is one of the main proteins found in the nectar of *N. benthamiana* and *N. clevelandii.* The major protein identified in nectar of *N. plumbaginifolia* was identified as Nectarin 5, spot 3, **Table 4**. The theoretical molecular weight was 59.8, very similar to obtained by SDS PAGE 60 kDa. The spots 4 from *N. langsdorffii* and 5 from *N. sylvestris* were identified as a Nectarin 3-like protein.

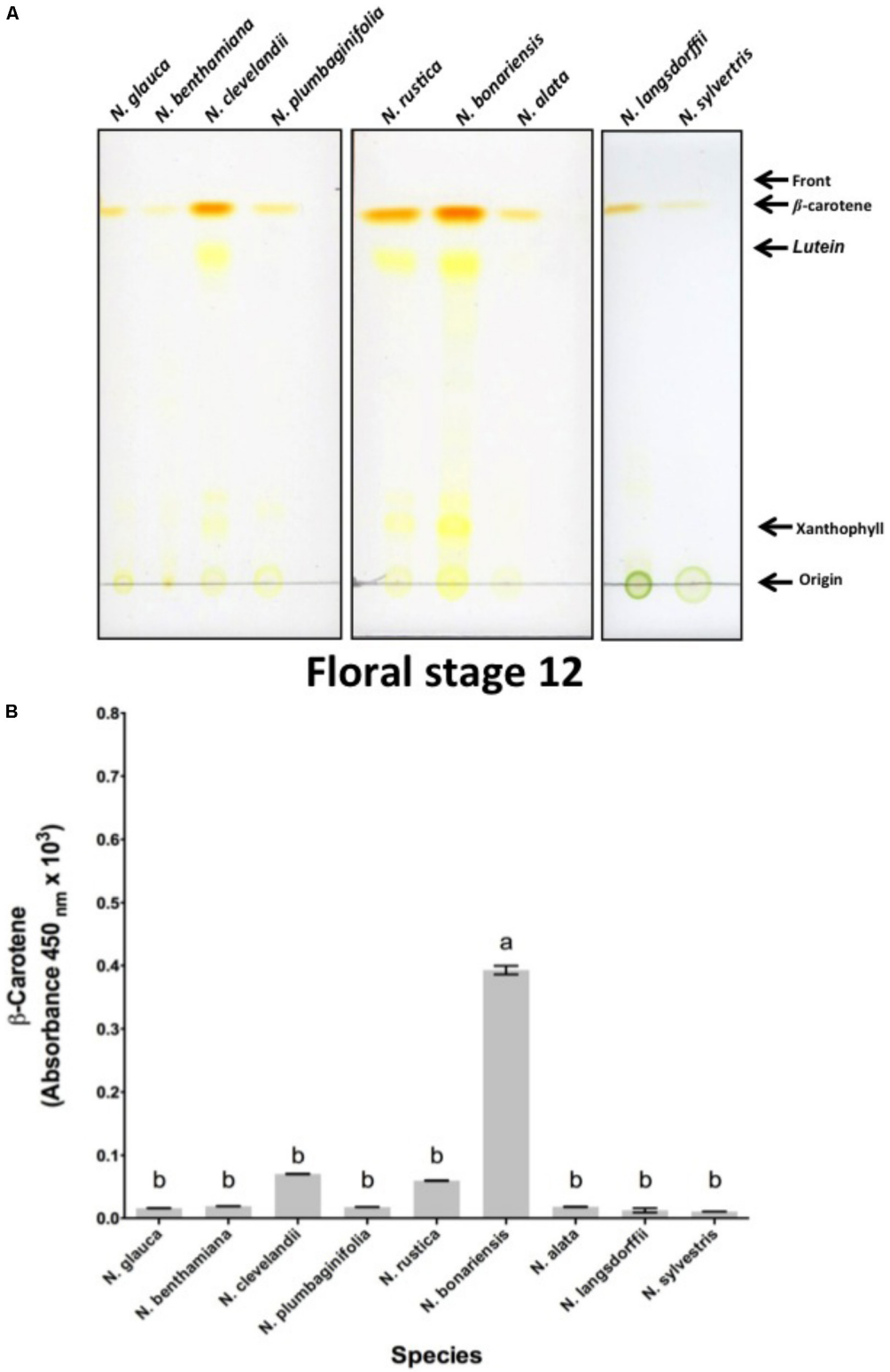

FIGURE 2 | Analysis of nectary-expressed carotenoids from different species of *Nicotiana* sp. **(A)** Silica gel thin-layer chromatogram (TLC) of carotenoids isolated from stage 12 nectaries of *N. glauca*; *N. benthamiana*; *N. clevelandii*; *N. plumbaginifolia*; *N. rustica*; *N. bonariensis* and *N. alata; N. langsdorffii;* and *N. sylvestris.* The origin, solvent front, and migration of various pigments are indicated. Solvent used was 9:1 acetone: hexane solvent. **(B)** Absorbance (450 nm) of β-carotene in nectary extracts at stage 12. Averages ± standard deviation ($N = 3$). Species evaluated: 1, *N. glauca*; 2, *N. benthamiana*; 3, *N. clevelandii*; 4, *N. plumbaginifolia*; 5, *N. rustica*; 6, *N. bonariensis;* 7, *N. alata;* 8, *N. langsdorffii;* and 9, *N. sylvestris.* Different letters mean statistical differences between groups.

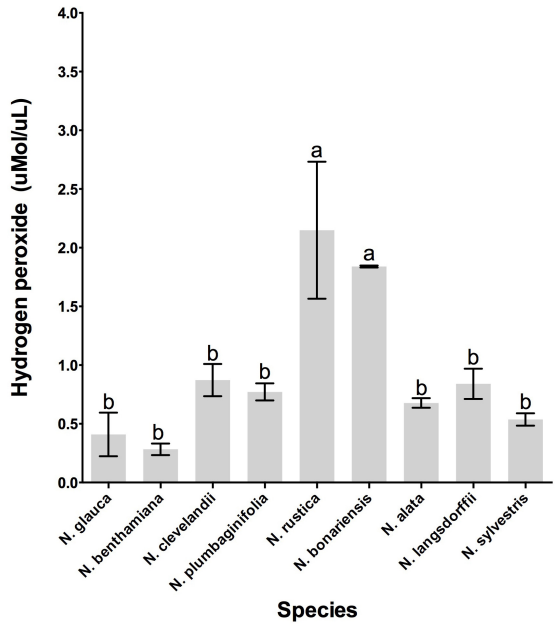

FIGURE 3 | Hydrogen peroxide production in the floral nectar of the selected *Nicotiana* species. Averages ± standard deviation (*N* = 3). Species evaluated: 1, *N. glauca*; 2, *N. benthamiana*; 3, *N. clevelandii*; 4, *N. plumbaginifolia*; 5, *N. rustica*; 6, *N. bonariensis;* 7, *N. alata;* 8, *N. langsdorffii*; and 9, *N. sylvestris.* Different letters mean statistical differences between groups.

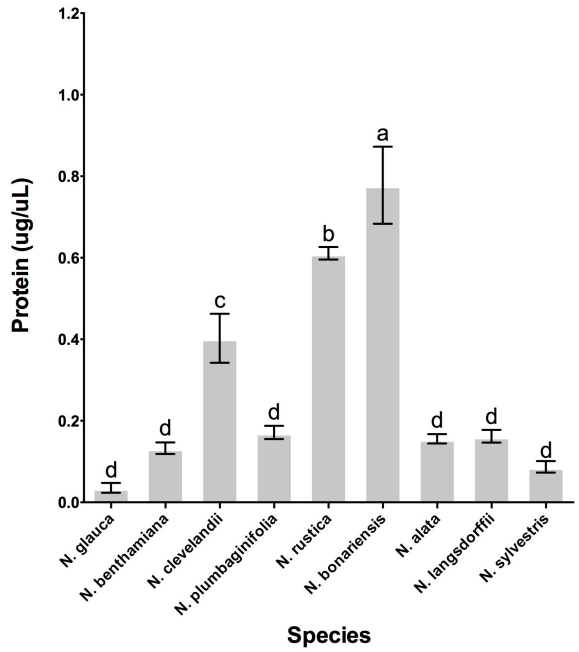

FIGURE 4 | Protein accumulation in the nectar of various *Nicotiana* sp. Species evaluated: 1, *N. glauca*; 2, *N. benthamiana*; 3, *N. clevelandii*; 4, *N. plumbaginifolia*; 5, *N. rustica*; 6, *N. bonariensis;* 7, *N. alata;* 8, *N. langsdorffii;* and 9, *N. sylvestris.* Different letters mean statistical differences between groups.

DISCUSSION

Because of previously observed significant differences between the nectars of the genus *Nicotiana* (Carter and Thornburg, 2004a) and the genus petunia (Hillwig et al., 2010a), we have investigated the nectars of a broad group of *Nicotiana* species to determine whether significant differences in nectars exist within this genus. To attract their pollinators, the plants offer floral nectar secreted into the floral tube at the base of the ovary that constitute a rich source of sugars, amino acids, vitamins and other ingredients which provides a rich reward to pollinators (Carter et al., 2006). However, the selected species used in this study include several different pollinator syndromes, **Table 1**.

It is known that several factors such as sugar composition, amino acids, organic acids and inorganic ions can affect the visitation of pollinators (Kessler and Baldwin, 2007; Afik et al., 2014; Tiedge and Lohaus, 2017). In addition, another important aspect as the floral biology can affect the access of the pollinators (Ackermann and Weigend, 2006). Thus, we conducted a study to understand the relationship between floral biology and the biochemistry of nectar from different genus of *Nicotiana*. As shown in **Table 2**, species like *N. benthamiana*, *N. clevelandii N. plumbaginifolia* showed intermediate flowers or in other species such as *N. alata* and *N. sylvestris* long flowers. Due to the floral characteristics these species have access to nectar more limited requiring specialized pollinators with long proboscis like hawkmoth. On the other hand, species like *N. rustica*, *N. bonariensis*, and *N. langsdorffii* showed short flowers indicating that nectar can be more easily accessed and has different composition. The nectar sugar concentration also differed among *Nicotiana* species, being divided into two groups. The sugars were higher in species with short flowers such as *N. bonariensis* and *N. langsdorffii* , while that other species showed lower concentrations (**Figure 1**). In species with intermediate or long flowers, there was no observed correlation between the floral length and the concentration

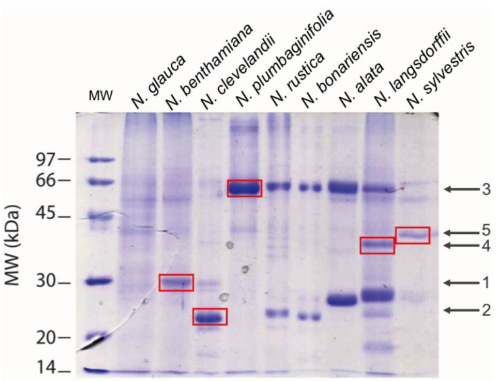

FIGURE 5 | Patterns of nectar proteins by SDS-PAGE 12.5%. In each lane was applied 1.25 μgP of raw nectar. Averages ± standard deviation. The standard profile was obtained from the analysis of three different independent experiments (*N* = 3). The proteins were stained with Coomassie Blue. Averages ± standard deviation (*N* = 3).

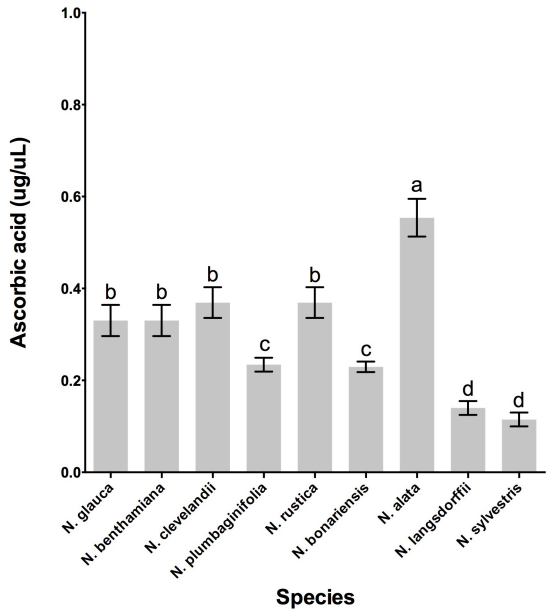

FIGURE 6 | Quantity of ascorbate from nectar of nine tobacco species flowers at stage 12. Averages ± standard deviation (*N* = 3). Species evaluated: 1, *N. glauca*; 2, *N. benthamiana*; 3, *N. clevelandii*; 4, *N. plumbaginifolia*; 5, *N. rustica*; 6, *N. bonariensis;* 7, *N. alata;* 8, *N. langsdorffii;* and 9, *N. sylvestris.* Different letters mean statistical differences between groups.

of sugar. Recently, (Tiedge and Lohaus, 2017) showed that this correlation is associated with the floral opening period. Day flowering *Nicotiana* species such as, *N. rustica and N. langsdorffii* show higher level of sugar than night flowering *Nicotiana* species. In addition of the floral biology, the nectar sugar composition is another factor that can significantly affect the visitation of pollinators (Torres and Galetto, 2002; Wolff, 2006; Witt et al., 2013). Sucrose represents one of the main sugars found in nectar (Chalcoff et al., 2006). The analysis of nectar carbohydrate composition from *Nicotiana* species **Table 3** showed that sucrose was the major sugar in floral nectar in all species analyzed. Three species, *N. alata, N. glauca,* and *N. sylvestris,* showed molar ratio (S/G + F) ≥ 1.00. From these species only *N. glauca* having intermediate-length flowers has been described as diurnal flowering species, while *N. alata* and *N. sylvestris* are night-flowering species. The higher sucrose to hexose molar ratio was previously shown in nectar of long night-flowering *N. alata* and *N. sylvestris* and common feature of night *Nicotiana* flowering species (Tiedge and Lohaus, 2017). The high content of sucrose in nectar of night-flowers or with long floral tubes is associated with higher starch storage in nectaries and different mechanisms of nectar secretion (Tiedge and Lohaus, 2017). Furthermore, the high content of sucrose is related to decrease in viscosity, which facilitates suction by pollinators with long proboscis (Nicolson et al., 2013; Tiedge and Lohaus, 2017). In addition, during the night with lower temperatures, the evaporation effect is reduced and is not necessary high osmolarity for nectar secretion. Thus, long flowered plants takes advantage of these conditions to secrete sucrose, a carbohydrate with low osmolarity (Witt et al., 2013). In addition to sucrose, the nectar of *Nicotiana* species also presented glucose and fructose in their composition and among the hexoses analyzed, fructose was the predominant sugar (**Table 3**). In some *Nicotiana* species studied, the fructose/glucose (F/G) molar ratio was higher or equal 1.0. However, an extremely high molar ratio (F/G) of 11.0 was observed in nectar of *N. glauca*, followed by *N. rustica*, with molar ratio of 10.0 (**Table 3**). Recently a high molar ratio (F/G) of 12.6 for nectar of *N. glauca* was described, suggesting that this feature is characteristic of this species. The high content of fructose in nectars has been associated with increase sweetness, thus increasing pollinator reward (Tiedge and Lohaus, 2017). Besides the floral characteristics and carbohydrate composition of the nectars, we also examined the biochemistry of nectary gland. In all species, the nectary gland increased in size and changed color as result of carotenoids accumulation Supplementary Figures S1–S4. Carotenoids (b-carotene) were observed in nectaries, and extreme levels were observed in nectaries of *N. bonariensis* **Figures 2A,B**. In nectaries, the production of carotenoids and ascorbate provides an antioxidant defense against the high level of hydrogen peroxide found in nectar (Horner et al., 2007). During development of nectaries, the high level accumulation of carotenoids in the nectaries starts about stage 9 when nectaries undergo a metabolic shift and starch are degraded to produce glucose. This glucose is then available to the methylerythritol phosphate (MEP) pathway, which leads to the production of IPP, the carotenoid precursor. The high levels of carotenoids are thought protect nectary cells from the severe oxidative processes that occur as a result of the Nectar redox cycle (Carter and Thornburg, 2004c).

In fact, the nectar of *N. bonariensis* showed one of the highest content of sugars and hydrogen peroxide (**Figure 3**). Thus, the high levels of carotenoids and other pigments such as lutein and xanthophyll may function as an additional defense to high level of hydrogen peroxide in nectar of this species. Similar observation was found in nectar of *N. rustica,* species with short flowers. In *N. rustica*, the hydrogen peroxide content showed the highest levels among all species tested (**Figure 3**), however, there appeared to be no correlation with the high levels of carotenoids or ascorbate. Although, *N. rustica* has intermediate sugar content, the short size flowers could facilitate the access pollinators and growth of microorganisms. Thus, the highest hydrogen peroxide content would be an additional nectar defense. The ascorbate is another important antioxidant involved in the Nectar Redox Cycle. Ascorbate was detected in nectar of all species, however, *N. alata* showed the highest levels (**Figure 6**). Ascorbate accumulates at high levels in nectaries at stage 12 (2 μg/nectary) (Horner et al., 2007), composing the nectar during the secretion process and integrating the Nectar Redox Cycle. As previously described, the Nectar Redox Cycle is the remarkable biochemistry pathway responsible for production of high levels of hydrogen peroxide in nectar. The SDS-PAGE analysis showed different profiles of the proteins in nectars **Figure 6**. *N. plumbaginifolia*, *N. langsdorffii*, *N. bonariensis,* and *N. alata* from Alatae section had similar profile. The proteomics analysis of the main protein in nectar of *N. plumbaginifolia* identified as Nectarin 5, spot 3, **Table 4**, being this protein was very evident Alatae section. This

TABLE 4 | Proteins from *Nicotiana* species nectars by ESI-Q-TOF MS/MS.

Spot No.	Theoretical		*PEP Score	**PSM	Identified peptides	Coverage (%)	Accession	Protein description
	MW (kDa)	pI						
#1	24.6	6.54	28.89	515	KVNGFPCKTNFTA HSKVKVNGFPCKT HPRASEMVFVMEG SEMVFVMEGELDV	24.89	Q94EG3	Nectarin-1
#2	24.6	6.54	5.59	42	IDYAPGGINPPHTHPR	6.98	Q94EG3	Nectarin-1
#3	59.8	8.60	15.93	329	KSMEEDLFWAIR	2.25	Q9SA89	Berberine bridge like enzyme (Nectarin-5)
#4	31.5	6.74	120.3	910	LVHESNNGKFVVI HLVHESNNGKFVV YDEKSENGPANWG SENGPANWGNIRP GPANWGNIRPDWK RPDWKECSGKLQS PSEHTINGERFNL TQYQLKQLHWHTP SLTTPPCTEGVVW HDGFETNARPTQP PDPFLSMIENDLK TNARPTQPENERY RPTQPENERYINS RQIKLLQEAVHDG	55.47	Q84UV8	Bifunctional monodehydro ascorbate reductase and carbonic anhydrase (Nectarin-3)
#5	31.5	6.74	28.30	35	LVHESNNGKFVVI HLVHESNNGKFVV SLTTPPCTEGVVW	28.30	Q84UV8	Bifunctional monodehydro ascorbate reductase and carbonic anhydrase (Nectarin-3)

**PEP score: Measures the significance of a single spectrum assignment with a specific PSM score. It is the probability of the PSM being incorrect, i.e., PEP of 0.01 means there is a 1% chance of the PSM being incorrect. **PSM: peptide-spectrum match, a spectrum that matches to a peptide sequence.*

is indicative that Nectarin 5 has a central role in the production of peroxide in Alatae section. Other species such as *N. glauca* and *N. sylvestris* had very little protein in their nectars, low abundance without majority proteins. In ornamental tobacco nectar, the nectarins are secreted as array of five proteins and accumulate to almost 250 mg/ml in nectar (Carter et al., 1999). The low abundance of proteins in the nectar *N. glauca*, *N. sylvestris,* and *N. benthamiana* may be associated the low content of hydrogen peroxide quantified these nectars. The very limited production of hydrogen peroxide suggesting that a different mechanism may exist for antimicrobial defense, as RNase activities described to petunia nectar (Hillwig et al., 2010a). Based upon these observations, we conclude that, although the oxidative processes that were first identified and characterized in the LxS8 interspecific cross, including the presence of hydrogen peroxide in nectar as well as antioxidants in both soluble nectar (ascorbate) and in nectary tissues (β-carotene) have been identified in all species, there are species-specific differences are found throughout the genus *Nicotiana*. Further, the major nectar proteins that we identified from these species belonged to the nectarin family of proteins (especially, Nec1, Nec3, and Nec5).

AUTHOR CONTRIBUTIONS

FS and RT designed the study, performed the biochemical assays, interpreted the experimental data, and wrote the manuscript. AG cultivated the plants and collected the samples used in all experiments. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01100/full#supplementary-material

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The Octadecanoid Pathway, but Not COI1, Is Required for Nectar Secretion in *Arabidopsis thaliana*

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Over 75% of crop species produce nectar and are dependent on pollinators to achieve maximum seed set, yet little is known about the mechanisms regulating nectar secretion. The phytohormone jasmonic acid (JA) is recognized to be involved in several plant processes including development and defense. JA was also recently shown to positively influence nectar secretion in both floral and extrafloral nectaries. For example, endogenous JA levels peak in flowers just prior to nectar secretion, but the details of how JA regulates nectar secretion have yet to be elucidated. We have found that the octadecanoid pathway does indeed play a role in the production and regulation of floral nectar in Arabidopsis. Null alleles for several JA biosynthesis and response genes had significantly reduced amounts of nectar, as well as altered expression of genes known to be involved in nectar production. We additionally identified crosstalk between the JA and auxin response pathways in nectaries. For example, the nectar-less JA synthesis mutant *aos-2* showed no auxin response in nectaries, but both nectar production and the auxin response were restored upon exogenous JA and auxin treatment. Conversely, *coi1-1*, a JA-Ile-insensitive receptor mutant, displayed no auxin response in nectaries under any circumstance, even in older flowers that produced nectar. Surprisingly, *opr3-1*, a mutant for *12-oxophytodienoate reductase* 3 [an enzyme further down the JA biosynthetic pathway that reduces 12-oxo phytodienoic acid (OPDA)], produced no nectar in newly opened flowers, but did secrete nectar in older flowers. Furthermore, a similar phenotype was observed in *coi1-1*. Cumulatively, these observations strongly suggest an indispensable role for an octadecanoic acid- and auxin-dependent, but JA- and COI1-dispensible, pathway in regulating nectar production in Arabidopsis.

Keywords: nectar, nectaries, jasmonic acid, auxin, F-box proteins, SWEET9

INTRODUCTION

Nectar is a major factor for enhancing plant-mutualist interactions. Nearly 90% of all plant species, including 75% of domesticated crops, benefit from animal-mediated pollination, which is usually facilitated by floral nectar (Klein et al., 2007; Calderone, 2012). Floral nectar is offered to increase pollinator visitation, while extra-floral nectar is used to attract mutualist insects that provide protection from herbivory (Heil, 2011). Despite the importance of nectar in attracting pollinators to promote outcrossing and help plants achieve maximum seed set, relatively little is known about the molecular regulation of nectar secretion.

Arabidopsis thaliana flowers, although highly self-fertile, surprisingly still produce nectar, which is thought to contribute to outcrossing events in natural populations (Hoffman et al., 2003; Kram and Carter, 2009). Due to the wealth of genomic resources and its close relatedness to agriculturally important relatives in the Brassicaceae family – that often require effective pollinator visitation for achieving maximum seed set – Arabidopsis has been an important model for studying the genetic and molecular mechanisms required for nectary function (Kram and Carter, 2009). Arabidopsis flowers have two types of nectaries: median and lateral. The lateral nectaries are located at the base of the short stamen and secrete >99% of total floral nectar (Davis et al., 1998; Kram and Carter, 2009). Median nectaries are located at the base of petals and long stamens and produce little to no nectar. Immature lateral nectaries accumulate starch, which is then broken down at anthesis and the resulting sugars are eventually secreted into the floral nectar (Stage 13–15; newly opened flowers, pollen shed and nectar secretion) (Ren et al., 2007; Kram and Carter, 2009). The nectar produced by these lateral nectaries of Arabidopsis (and most Brassicaceae species) is hexose-rich (nearly all glucose and fructose) (Davis et al., 1998).

A few recent reports have enhanced our understanding of nectar production. The current literature increasingly supports an eccrine-based secretion model for floral nectar in the Brassicaceae (Roy et al., 2017). Eccrine-based secretion relies on plasma membrane-localized pores and transporters to export nectar metabolites from parenchymal cells in the nectary. In one model, nectary starch is degraded and re-synthesized into sucrose by sucrose phosphate synthases (SPS) and other enzymes (Lin et al., 2014). Next, the sucrose is exported into the apoplastic space via the sucrose uniporter AtSWEET9 (Lin et al., 2014). In the apoplastic space CELL WALL INVERTASE 4 (AtCWINV4) catalyzes the hydrolysis of sucrose into hexose monomers, glucose and fructose (Ruhlmann et al., 2010). This invertase activity creates both a constant driving force for sucrose export and a negative water potential causing water to move toward sugars and create water droplets. Perhaps not surprisingly, knockout mutants of *SPS, SWEET9,* and *CWINV4* all lack nectar production. Although we are beginning to understand key genes involved in the process of nectar secretion, the ways in which these processes are regulated is also still poorly understood.

In order to support effective mutualist visitation and proper pollination, floral nectar production must be carefully coordinated with petal opening, pollen shed, stigma receptivity and pollinator activity. Therefore, it is not surprising that floral nectar production would require hormonal regulation to ensure its production is tightly coordinated with these other important processes. Even though proper regulation of nectar secretion is essential to its overall function – effectively manipulating pollinator visitation (Pyke, 2016) – the impacts of each of the phytohormones has remained rather elusive in relation to nectar production. Some studies have examined the relative impacts of auxin (IAA, indole acetic acid) (Bender et al., 2013), gibberellins (Wiesen et al., 2016), and jasmonic acid (JA) (Radhika et al., 2010) with regard to their roles in regulating floral nectar secretion as outlined below.

It is well established that auxin is an important phytohormone that heavily regulates both developmental processes as well as responses to biotic and abiotic stresses (Chapman and Estelle, 2009; Lokerse and Weijers, 2009; Leyser, 2010; Zhao, 2010; Weijers et al., 2018). IAA activates transcriptional responses through binding to the TIR1 F-box receptor, which leads to the ubiquitin-mediated degradation of AUX/IAA transcriptional repressors and the de-repression of auxin response factors (ARFs) thus activating auxin response genes (Dharmasiri et al., 2005). IAA is important for regulating proper floral development too (Aloni et al., 2006). Furthermore, recent reports have shown that auxin-related genes display nectary-enriched expression profiles in the Brassicaceae (Kram et al., 2009; Hampton et al., 2010). Interestingly, *PIN6*, an auxin efflux transporter, was shown to have a positive effect on total nectar production as *PIN6* overexpressers showed an increase in total nectar production and *PIN6* knockdown mutants show a decrease in nectar production in Stage 14–15 flowers (Bender et al., 2013). When Arabidopsis inflorescences were treated with exogenous auxin there was a 2 – 10-fold increase in total nectar production in Arabidopsis and *Brassica napus*, whereas the auxin transport inhibitor NPA decreased nectar output (Bender et al., 2013).

In addition to IAA, JA is another phytohormone known to play important roles in plant flower development as well as plant defense (Wasternack and Hause, 2013). JA is a lipid-derived hormone whose biosynthesis occurs via the octadecanoic pathway and begins with the cleaving of alpha-linolenic acid (C18:3) off of lipid bilayers by phospholipases (e.g., Ishiguro et al., 2001) (see **Supplementary Figure S1** for diagram of full synthetic pathway). The primary mode of JA signaling occurs through a similar mechanism as described for IAA. JA signals through the COI1 F-box protein that forms a Skp-Cullin-F-box (SCF) ubiquitin ligase complex that leads to the ubiquitination and degradation of JAZ repressors in the presence of jasmonoyl-L-isoleucine (JA-Ile), the bioactive form of JA (Kelley and Estelle, 2012; Nagels Durand et al., 2016). Some previous reports have shown that JA plays a role in the regulation of nectar production. For example, JA levels in *B. napus* flowers peak just prior to anthesis which, as previously mentioned, is coincidental with the onset of nectar production (Radhika et al., 2010). Furthermore, exogenous floral application of phenidone, a chemical inhibitor of JA synthesis, lowered nectar production while exogenous JA increased total nectar (Radhika et al., 2010). In tobacco, JA's importance in regulating nectar secretion is also evident. Tobacco flowers silenced for JA synthesis and response have no nectar and show altered starch utilization (Liu and Thornburg, 2012; Wang et al., 2014). For example, the JA-responsive transcription factor NtMYB305 was shown to be required for nectary maturation and nectar secretion (Liu et al., 2009; Liu and Thornburg, 2012; Wang et al., 2014). These mutants also showed defects in starch accumulation. Furthermore, Stitz et al. (2014) showed that JA-Ile signaling through COI1 is required for nectar production in tobacco flowers. Although there is a small body of work implicating that JA and auxin are involved in nectary maturation and nectar secretion, the details as to exactly how these hormones regulate these processes is still an area that requires further investigation.

Phytohormones do not work in isolation and the coordinated interactions between JA, IAA, and GA make it difficult to study the sole influence that each of these hormones may have on nectary function. For instance, in Arabidopsis IAA acts through ARF6 and ARF8 to induce JA synthesis leading to the expression of *MYB21* and *MYB24* which together play important roles in flower maturation (Nagpal et al., 2005; Reeves et al., 2012). To add to the complexity of this hormonal coordination, gibberellin-deficient flowers show reduced levels of JA and lower expression of *MYB21/24* (Cheng et al., 2009). Using combinations of JA biosynthesis and response mutants, in addition to transgenic lines with altered auxin levels, here we demonstrate that the crosstalk between JA and IAA is essential for the regulation of nectary function.

MATERIALS AND METHODS

Plant Material and Growth Condition

All plants were grown on peat-based medium with vermiculite and perlite (Pro-Mix BX; Premier Horticulture) in individual pots. Arabidopsis seeds were sterilized in a 33% bleach solution + 0.01% Triton mix for 10 min and subsequently washed five times with water before being planted. The plants were either housed in a Percival AR66LX growth chamber with 16 h day/8 h night, photosynthetic flux of 150 μmol m^{-2} sec^{-1} and at 23°C or in a growth room with same 16 h day/8 h night cycle at 22°C. It should be noted that plants used for any direct comparisons were grown at the same time, in the same trays, under the same growth conditions to limit environmental effects on nectar production. Arabidopsis seed for wild-type (Col-0), *dad1-1* (SALK_138439), and *jar1-11* (CS67935) were obtained from the Arabidopsis Biological Resource Center (ABRC). DR5::GUS plants were previously described (Ulmasov et al., 1997). Homozygous mutants of *myb21-4, opr3-1*, *coi1-1*, and *aos-2* (SALK_017756) were provided by the Reed Group at the University of North Carolina at Chapel Hill (Reeves et al., 2012). Both *coi1-1* and *myb21-4* are sterile and not rescuable via exogenous JA application, as such they were carried as heterozygous mutants by backcrossing to wild-type Col-0 and subsequent selection for homozygous mutants in downstream experiments. Jas9-VENUS (Stock N2105629) and mJas-9 Venus (Stock N2105630) seeds were ordered from Nottingham Arabidopsis Stock Center (NASC) and previously described by Larrieu et al., 2015. All T-DNA mutants were genotyped with gene specific primers (**Supplementary Table S1**) flanking the T-DNA insertion site and the T- DNA specific primer "LBb1.3" to screen for the T-DNA insert as described at: http://signal.salk.edu/tdnaprimers.2.html.

Plants expressing the auxin synthesis gene *iaa*M under the control of the nectary-specific SWEET9 promoter were generated by PCR amplifying the *iaa*M gene out of *Pseudomonas savastanoi* genomic DNA using the primer pair "*iaaM* ORF-F" and "*iaaM* ORF-R" and then ligating it into the *Xma*I and *Sme*I sites downstream of the nectary-specific SWEET9 promoter in the plant transformation vector pPMK1 (Bender et al., 2012). This vector was subsequently given the name pPMK21. *Agrobacterium tumefaciens* (GV3101) cells were transformed to carry the pPMK21 vector and used to transform Arabidopsis using the floral-dip method described by Clough and Bent (1998). Transformed seedlings were selected on one half Muarshige and Skoog medium plates with 50 μg/ml kanamycin.

Hormone Treatment of Flowers

1-naphthalene acetic acid (1-NAA) (Cat #N0640) and methyl jasmonate (Cat #392707) were obtained from Sigma-Aldrich. 1-NAA stocks were prepared in ethanol. Treatment of flowers with 10 μM 1-NAA or 500 μM methyl jasmonate was conducted by dipping the inflorescence tips in the appropriate hormone solution dissolved in a 0.05% aqueous Tween 20 solution the evening before testing nectar production. Flowers were phenotyped for the production of nectar and anther dehiscence, or RNA was isolated via the protocol described below.

Gene Expression Analysis

For qRT PCR analysis, RNA was isolated from tissues (minimum of 10 flowers per biological replicate) with the Absolutely RNA Miniprep kit according to the manufacturer's instructions (Agilent, Catalog #400800). RNA quality was confirmed by spectrophotometric analysis and agarose gel electrophoresis. For cDNA preparation, total RNA (500–1,000 ng) was used as template for Promega's Reverse Transcription System (Promega, Catalog #A3500). 10 ng of the resulting cDNA was added to the real time PCR reaction mix, which included 10 μl of 2× Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Cat #600880), 0.4 μl of each forward and reverse primer (10 μM stock), 0.4 μl ROX dye (high), and 6.8 μl nuclease-free H2O. Primers were designed using the online primer design tool "QuantPrime." The Applied Biosystems StepOnePlus thermocycler was used for real-time PCR, and results were analyzed with Applied Biosystems StepOne software (v2.3). Three biological replicates with a minimum of two technical replicates were performed for each experiment.

Histochemical Staining for Starch and GUS

Starch staining was performed as described by Ruhlmann et al. (2010). Freshly collected flowers were dipped in Lugols iodine solution (Fischer, Cat # S93408) for 5 min after a 1-min vacuum infiltration in the same. Sepals were removed carefully before beginning staining to improve stain permeation. Flowers were subsequently rinsed in deionized water twice to wash off excess stain and subsequently imaged under a dissecting microscope. GUS staining was performed as described previously (Jefferson et al., 1987). For GUS staining, flowers were dissected to remove sepals and subsequently dipped in a GUS staining solution, kept under 15 psi vacuum for 15 min and incubated at 37°C for 16 h. The stain was subsequently removed and the flowers were washed with 90% ethanol thrice, with each wash lasting 2–3 h. The final wash was with 70% ethanol overnight. The flowers were then transferred to deionized water and imaged under a dissecting microscope.

Microscopic Analysis

Flowers expressing mJas9-Venus or Jas9-Venus were viewed either using an Olympus BX-53 fluorescence microscope (YFP cube) or a Nikon A1si spectral confocal microscope attached to a Nikon TE2000 motorized inverted microscope (Nikon USA, Melville, NY, United States) using the 514 nm laser line (laser power 25.4). The emitted fluorescence signal was collected using the 32-channel spectral detector at 6.0 nm spectral resolution using the 20× objective (n.a. 0.75) with zoom setting of 4. Optical sections were collected at 0.5 μm increments then spectrally unmixed using Nikon Elements software ver. 5.1.2.

RESULTS

Exogenous Methyl Jasmonate Induces Nectar Secretion in Arabidopsis JA Synthesis Mutants

Jasmonic acid has been suggested to play a role in nectar production (Heil, 2001; Radhika et al., 2010; Wang et al., 2014). In order to examine the role of JA in Arabidopsis nectar production, we first observed mutants of the JA biosynthesis genes *DELAYED ANTHER DEHISCENCE* (*DAD1*, AT2G44810) and *ALLENE OXIDE SYNTHASE* (*AOS*, AT5G42650). *AOS* is a single copy gene indispensable for JA synthesis (Park et al., 2002), whereas *DAD1* encodes a filament-specific phospholipase involved in JA synthesis in flowers, but plants are not entirely JA deficient (Ishiguro et al., 2001). The first two open flowers (equivalent of Stage 14 in Col-0) of both *aos-2* and *dad1-1* produced no nectar while appearing to maintain proper nectary morphology (**Figures 1B,C**). The mutants also exhibited male sterility (data not shown), which was a previously known phenotype to each mutant (Ishiguro et al., 2001; Park et al., 2002). When these synthesis mutants were exogenously treated with the volatile methylated form of JA, methyl jasmonate (MeJA), nectar production was rescued in recent fully opened flowers (equivalent to Stage 14 flowers in wild-type; **Figures 1E,F**). Wild-type plants showed no obvious change in nectar volume in response to MeJA treatment (**Figure 1D**). Since JA synthesis mutants are sterile, their flowers do not quickly dehisce like wild-type, instead their 'older' flowers (third open flower and down from the meristem) remain open. Interestingly, these 'older' open flowers in *dad1-1* produced nectar, whereas those of *aos-2* did not (**Supplementary Figures S1, S2**). The relationship between nectar secretion in 'young' and 'old' flowers of JA synthesis and response mutants is further explored below. Cumulatively, these results further support an essential role for JA in regulating nectar production.

Exogenous Methyl Jasmonate and Auxin Induce Nectar Secretion and Auxin Responses in *aos-2* Nectaries

Bender et al. (2013) previously showed that auxin homeostasis is important for nectar secretion. Cross talk between auxin and JA is also important for floral development (Nagpal et al., 2005; Varaud et al., 2011; Reeves et al., 2012), therefore we hypothesized that JA may be involved in crosstalk with auxin to regulate nectary function and an altered auxin response would be observed in the JA mutants. To further explore this hypothesis, *aos-2* was crossed into the auxin-responsive DR5::GUS reporter line and whole flowers were subjected to histochemical GUS staining (Ulmasov et al., 1997). In the wild-type background the DR5::GUS reporter showed a strong auxin response in both the median and lateral nectaries (**Supplementary Figure S2A**). However, there was no signal in the nectaries of the *aos-2* background, which produce no nectar (**Figures 2A,D**). When flowers were treated with MeJA, both nectar production and the nectary auxin response were rescued (**Figures 2B,E**).

Since previous reports have shown auxin treatment can increase nectar production (Bender et al., 2013), we decided to test if exogenous application of 1-naphthaleneacetic acid (NAA) – a synthetic membrane permeable auxin – could rescue nectar production in JA mutants. As with MeJA, floral crowns exogenously treated with 10 μM NAA displayed a restoration of nectar production (**Figure 2C**).

Given the dramatic change of nectar production in these JA biosynthesis mutants, we examined the expression of previously characterized genes known to be involved in nectar production in *aos-2* flowers exogenously treated with either MeJA or NAA (**Figure 2G**). Genes targeted for qRT-PCR analysis in JA mutants included *SWEET9*, a sucrose transporter (Lin et al., 2014), *CWINV4*, an invertase responsible for cleaving sucrose into hexose sugars (Ruhlmann et al., 2010), *PIN6*, an auxin efflux transporter (Bender et al., 2013) and *MYB21*, an ortholog of the JA-inducible tobacco transcription factor MYB305, which is required for nectar secretion (Liu et al., 2009; Liu and Thornburg, 2012; Reeves et al., 2012; Wang et al., 2014).

SWEET9 and *PIN6* transcripts were downregulated in nectarless mock treated *aos-2* flowers relative to Col-0 (wild-type) (**Figure 2G**), which in itself could explain the loss of nectar production (Bender et al., 2013; Lin et al., 2014). Exogenous treatment of MeJA restored nectar production (**Figure 2E**) and induced the expression of *SWEET9*, *CWINV4*, and *MYB21* by ∼2-fold compared to wild-type and ∼4-fold relative to the mock treated *aos-2* specifically for *SWEET9* (**Figure 2G**). *aos-2* flowers treated with exogenous NAA also produced nectar and restored *SWEET9* transcript to wild-type levels, but *MYB21* transcript abundance was downregulated and had little effect on *PIN6* transcript level relative to mock-treated Col-0 (**Figure 2G**).

Nectary-Derived Auxin Can Rescue Nectar Secretion in *aos-2*

Exogenous auxin (NAA) restored nectar production in *aos-2* flowers (**Figure 2**), but since NAA could only be applied to whole flowers it was not clear if the auxin-dependent signaling was limited to the nectaries. Thus, we decided to engineer transgenic *aos*-2 that could produce high endogenous auxin specifically in the nectaries by expressing the auxin biosynthesis gene *iaa*M (Klee et al., 1987) under control of the nectary-specific SWEET9 promoter (Bender et al., 2013; Lin et al., 2014). This construct, SWEET9pro::*iaa*M, was first transformed into wild-type plants. The subsequent SWEET9pro::*iaa*M transgenic line was then crossed into *aos-2* and phenotyped. The first two fully open

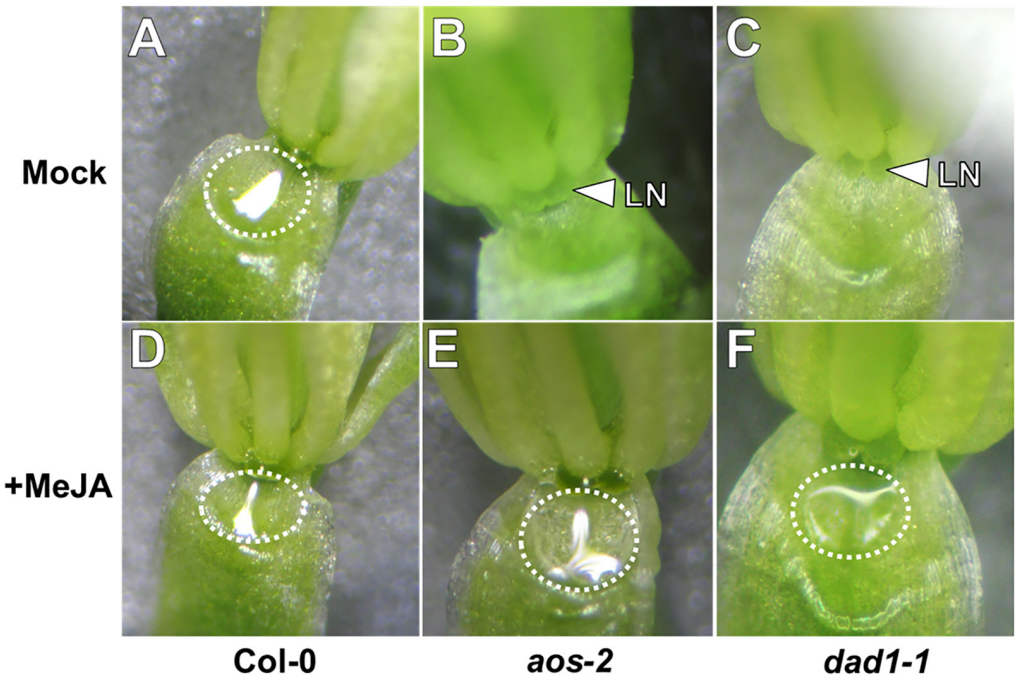

FIGURE 1 | Exogenous methyl jasmonate induces nectar secretion in Arabidopsis JA synthesis mutants. Mock treated wild-type flowers at Stage 14 (fully open) secrete nectar **(A)**, whereas the equivalent flowers of JA synthesis mutants (*aos-2*, **B** and *dad1-1*, **C**) do not. Exogenous application of MeJA (500 μM; **D–F**) induces nectar secretion in JA synthesis mutant flowers **(E,F)**. Arrowheads indicate the location of the lateral nectaries (LN) and dashed circles outline the presence of nectar droplets.

flowers ('young,' **Figure 3A**) of *aos-2* x SWEET9pro::*iaa*M did not produce nectar (**Figure 3C**) similar to both the 'young' and 'old' flowers of *aos-2* alone (**Figures 1B**, **3B**). The 'old' flowers (open flowers 3–5 of down from the meristem) of *aos-2* × SWEET9pro::*iaa*M did produce nectar (**Figure 3D**). Hence the nectary-derived auxin eventually caused nectar to be produced in the *aos-2* background. It should be noted that the anthers never dehisced in *aos-2*, either with or without SWEET9pro::*iaa*M, so fertilization was not a cause of the auxin signaling.

To determine if gene expression could account for nectar secretion observed in *aos-2* × SWEET9pro::*iaa*M, we first verified that *iaa*M expression was induced in 'old' vs. 'young' flowers (**Figure 3E**). We hypothesized that the low expression of *SWEET9* in the *aos-2* background (**Figure 2G**) would correspondingly keep *iaa*M levels low in younger *aos-2* × SWEET9pro::*iaa*M flowers. The gradual increase in endogenous auxin, driven by control of the SWEET9 promoter, subsequently would result in a positive feedback loop and nectar production in 'old' *aos-2* × SWEET9pro::*iaa*M flowers. Indeed, *SWEET9* transcripts in *aos-2* × SWEET9pro::*iaa*M flowers were significantly downregulated in 'young' flowers, but upregulated >3-fold in 'old' flowers relative to Stage 14 Col-0 flowers (open and secreting nectar) (**Figure 3F**). As a control, flowers from both 'young' and 'old' *aos-2* not harboring the SWEET9pro::*iaa*M transgene were examined for *SWEET9* expression, which demonstrated that SWEET9 is not induced in 'old' flowers not carrying *iaa*M (**Supplementary Figure S3**), suggesting that SWEET9 expression in *aos-2* × SWEET9pro::*iaa*M flowers is dependent on endogenous auxin synthesis. These results cumulatively support the hypothesis that endogenous auxin can rescue *aos-2* nectar secretion phenotypes downstream of JA.

The JA Receptor COI1 Is Not Required for Nectar Secretion, but Is Required for the Nectary Auxin Response

In light of the apparent dependence of nectar secretion on JA, an interesting phenotype was observed in the 'old' flowers of the JA-biosynthesis mutants *dad1-1* and *opr3-1*. Specifically, 'young' *dad1-1* and *opr3-1* flowers behaved similarly to those of the JA-deficient *aos-2* by not producing nectar, but their 'old' flowers secreted large nectar droplets (**Supplementary Figure S2B** and **Figure 4A**), unlike older *aos-2* flowers (**Figure 3B**). However, these results should be considered in the context of the fact that neither *dad1-1* nor *opr3-1* are completely JA-deficient [there is partial functional redundancy in the case of *DAD1* (Ishiguro et al., 2001)] and *opr3-1* is a leaky mutant (Chehab et al., 2011). Regardless of this fact, 'young' nectarless flowers crossed with DR5::GUS displayed no nectary auxin response, whereas the older flowers with nectar did have a robust auxin response specifically in the nectaries, although only in the median nectaries in the case of *opr3-1* (**Figure 4A** and **Supplementary Figure S2B**), again reinforcing a connection between auxin signaling and nectar secretion downstream of JA.

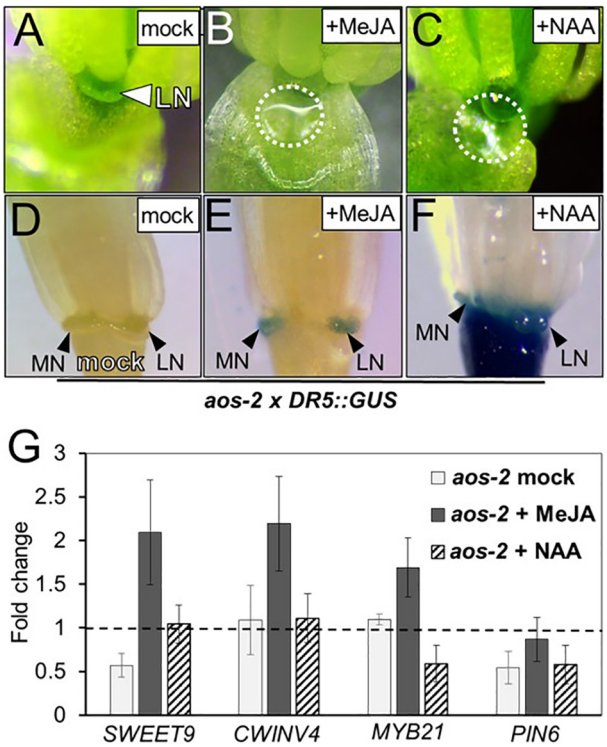

FIGURE 2 | Exogenous methyl jasmonate and auxin induce nectar secretion and auxin responses in *aos-2* nectaries. Mock treated *aos-2* flowers do not secrete nectar **(A)** and lack the nectary auxin response **(D)** observed in wild-type plants. Treatment of *aos-2* flowers with either MeJA (500 μM) or the synthetic auxin NAA (10 μM) induces nectar secretion **(B,C)** and the nectary auxin response **(E,F)**. The expression of key genes involved in nectar production were evaluated in whole *aos-2* Stage 14 flowers after treatment with MeJA or NAA by qRT-PCR **(G)**. Data is presented as fold-change in expression of each gene normalized to Col-0 (dashed line).

Previous reports have shown that the F-box protein CORONATINE INSENSITIVE 1 (COI1), which is a component of a JA receptor critical for JA-mediated signaling, is indispensable for floral starch metabolism and nectar secretion in tobacco flowers (Wang et al., 2014). Therefore, in addition to our JA biosynthesis mutants, we examined the reportedly JA-insensitive *coi1-1* in Arabidopsis. As also observed in *aos-2* × SWEET9pro::*iaaM*, *dad1-1* and *opr3-1*, there was a distinct difference in floral nectar secretion between 'young' and 'old' flowers. 'Young' *coi1-1* flowers (equivalent to Stage 14 in Col-0), showed a nectar-less phenotype, whereas the 'old' flowers did produce nectar (**Figure 4B**), suggesting the presence of a COI1-independent pathway responsible for regulating certain aspects of nectar secretion in Arabidopsis. Further support for the notion of a COI1-dispensible route for nectar secretion comes from the fact that both exogenous NAA and MeJA induced nectar secretion in 'young' *coi1-1* flowers (**Supplementary Figure S4**).

We also examined a mutant of *JASMONATE RESISTANT 1 (JAR1)* (AT2G46370), *jar1-11*, which is deficient in the JAR1 enzyme that conjugates JA to isoleucine in order to generate the bioactive JA-Ile. JA-Ile interacts directly with COI1 to mediate JA signal transduction (Staswick and Tiryaki, 2004). Interestingly *jar1-11*, which has previously been shown to accumulate JA-Ile levels to only 10% of that in WT upon wounding (Suza and Staswick, 2008), still produced nectar (**Supplementary Figure S5**) suggesting that either the low levels of JA-Ile are adequate for signaling nectar production or that there may be an alternative signaling module involved for nectar secretion.

There were notable changes in the expression of essential genes required for nectar production in the *aos-2* mutant that may account for the absence of nectar (**Figures 2**, **3**). We thus hypothesized that a similar pattern of gene expression may be observed in *opr3-1* and *coi1-1* that may cause the 'young' and 'old' nectar phenotype. Young nectarless *opr3-1* and *coi1-1* flowers showed a strong reduction in the expression of *SWEET9* (required for nectar production), whereas older flowers with nectar displayed a ∼3-to-4-fold induction of *SWEET9*, *CWINV4,* and *MYB21* relative to wild-type) (**Figures 4C,D**). Interestingly, *PIN6* expression begins 3.5-fold higher in 'young' *opr3-1* flowers but drops off to wild-type levels in 'old' flowers (**Figure 4C**). No significant difference in *PIN6* (auxin transporter) expression was observed in either the 'young' or 'old' flowers of *coi1-1* relative to Col-0 (**Figure 4D**). However, *coi1-1* × DR5::GUS failed to display an auxin response in either 'young' (no nectar) or 'old' (with nectar) flowers (**Figure 4B**). These results imply the presence of a COI1-independent pathway for nectar secretion, but that COI1 is required for the auxin responses observed in the nectaries of other JA-biosynthesis mutants.

JA Responses Are High in Nectaries During Nectar Production as Revealed by a Biosensor

A recently developed JA reporter system can be used to observe jasmonate signaling *in planta* (Larrieu et al., 2015). In brief, this system was created based on the knowledge that the Jas motif in JAZ proteins is required for their degradation upon JA-induced signaling, thus releasing the repression of JA-inducible genes (Yan et al., 2009; Wasternack and Hause, 2013). The Jas motif of AtJAZ9 was fused with VENUS, a fast maturing YFP and placed under the control of the CaMV 35S promoter. These lines were designated as Jas9-VENUS and robust studies confirmed their use as a JA biosensor (Larrieu et al., 2015). A mutant version, mJas9-VENUS, was also engineered such that it cannot be targeted for degradation by COI1, even in the presence of bioactive JA-Ile (Larrieu et al., 2015). Thus, Jas9-VENUS is rapidly degraded in tissues undergoing active JA responses and an be monitored by a reduction in fluorescence, whereas stabilized mJas9-VENUS is not degraded.

We hypothesized that the JA signaling and response in the nectaries of a Stage 14 flower would be high and thus lead to active degradation of Jas9-VENUS, manifesting as no or very low signal in a Jas9-VENUS line whereas the mJas9-VENUS line would show a nuclear signal in the nectaries. To confirm that the plants being tested indeed had a VENUS-dependent fluorescence signal, 5-day-old seedlings grown on half strength MS media were imaged with a fluorescence microscope. Seedlings with a positive signal for both Jas9- and mJas9-VENUS signal in the

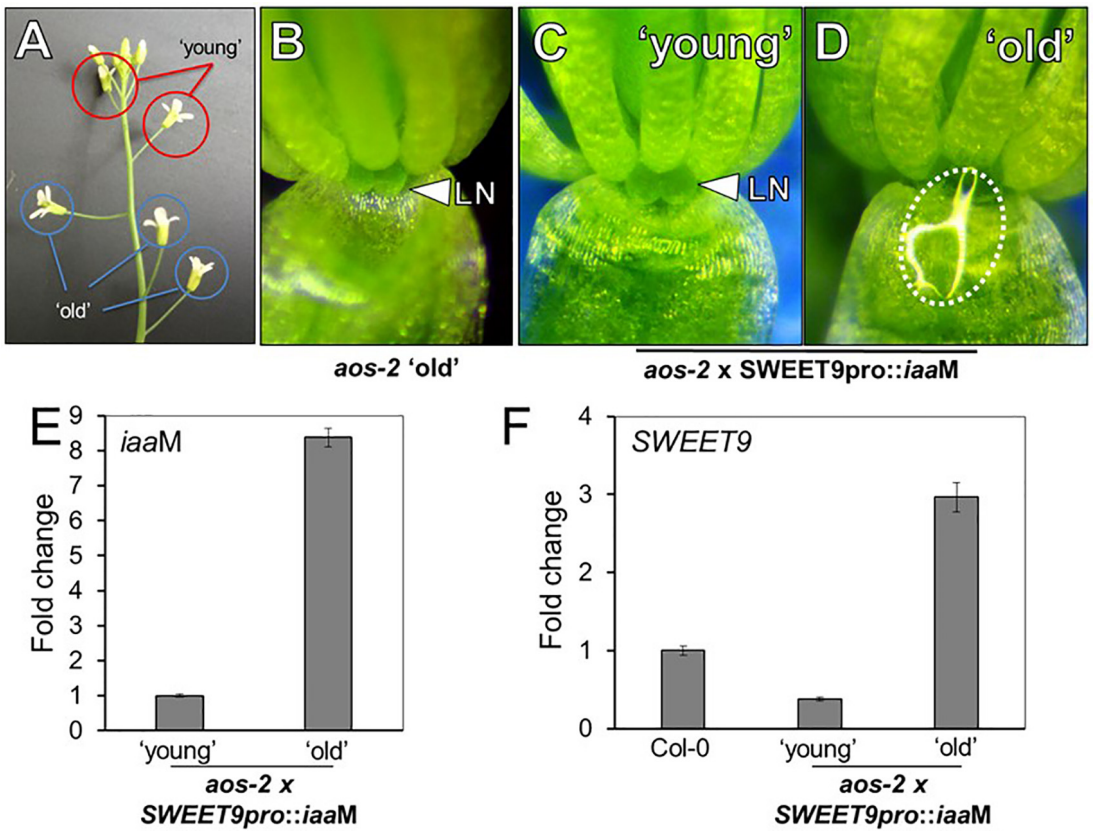

FIGURE 3 | Endogenous auxin induces nectar secretion in *aos-2*. **(A)** Relative locations of 'young' and 'old' flowers in JA-synthesis mutants, like *aos-2*. 'Old' *aos-2* flowers produced either no nectar **(B)** or a very faint glistening of fluid on the inner surface of the sepal. 'Young' *aos-2* flowers expressing the auxin synthesis gene *iaa*M under control of the SWEET9 promoter do not produce nectar **(C)**, whereas the corresponding 'old' flowers produce large nectar droplets **(D)**. **(E)** *iaa*M expression is strongly induced in 'old' *aos-2* × SWEET9pro::*iaa*M flowers (qRT-PCR data normalized to 'young' flowers). **(F)** Expression of *SWEET9* transcripts in Col-0 and 'young' and 'old' *aos-2* × SWEET9pro::*iaa*M flowers (qRT-PCR data normalized to Col-0 flowers).

roots (**Figures 5E–H**) were transplanted to soil. Fluorescence imaging of Stage 14 flowers reveal a strong signal in the nuclei of the nectaries of the mJas9-VENUS lines whereas the signal was absent in the Jas9-VENUS nectaries (**Figures 5A–D**) suggesting that JA signaling was active in nectaries during nectar production. To confirm that the signal was not due to autofluorescence we imaged Stage 14 mJas9-VENUS flower nectaries with a confocal microscope and spectrally unmixed the image to differentiate VENUS from autofluorescence (**Supplementary Figure S6**). These results indicate that JA is acting as primary signal directly in the nectaries and not in other proximal floral tissues that may be transmitting secondary signals to the nectaries.

MYB21 Is Required for Nectar Production

The transcription factor MYB21 is JA inducible and has been shown to play a critical role in stamen maturation and overall flower development (Stintzi and Browse, 2000; Stracke et al., 2001; Mandaokar et al., 2006; Cheng et al., 2009; Song et al., 2011). More specifically to nectar regulation, MYB305, the tobacco ortholog of MYB21, was reported to directly play a role in nectar production through the regulation of starch metabolism in tobacco flowers (Liu and Thornburg, 2012; Wang et al., 2014). *MYB305* mutants accumulate lower levels of starch in their nectaries and have reduced levels of nectar production (Liu and Thornburg, 2012). Reeves et al. (2012) also showed that 20 nectary specific genes were down-regulated in the *myb21-5 myb24-5* double mutant. Even with these reports in the literature, there has yet to be a report on the role of *MYB21* in Arabidopsis nectary function.

myb21-4 is a null mutant previously described as having a premature stop codon (Trp116*) (Reeves et al., 2012). As might be expected, the flowers of these mutants produce no nectar (**Figure 6A**). Perhaps unsurprisingly, nectar production was not rescued by exogenous application of MeJA (**Figure 6B**) since MYB21 is required downstream of JA signaling (Reeves et al., 2012). To further elucidate what genes MYB21 may be regulating we examined the expression of *SWEET9*, *CWINV4,* and *PIN6* via qRT-PCR analysis. All three genes showed a severe reduction in expression (>80%) (**Figure 6C**). Our data supports that the activity of MYB21 in the floral nectary indeed works downstream of JA and has a critical role in regulating the expression of several genes known to be essential for nectar production.

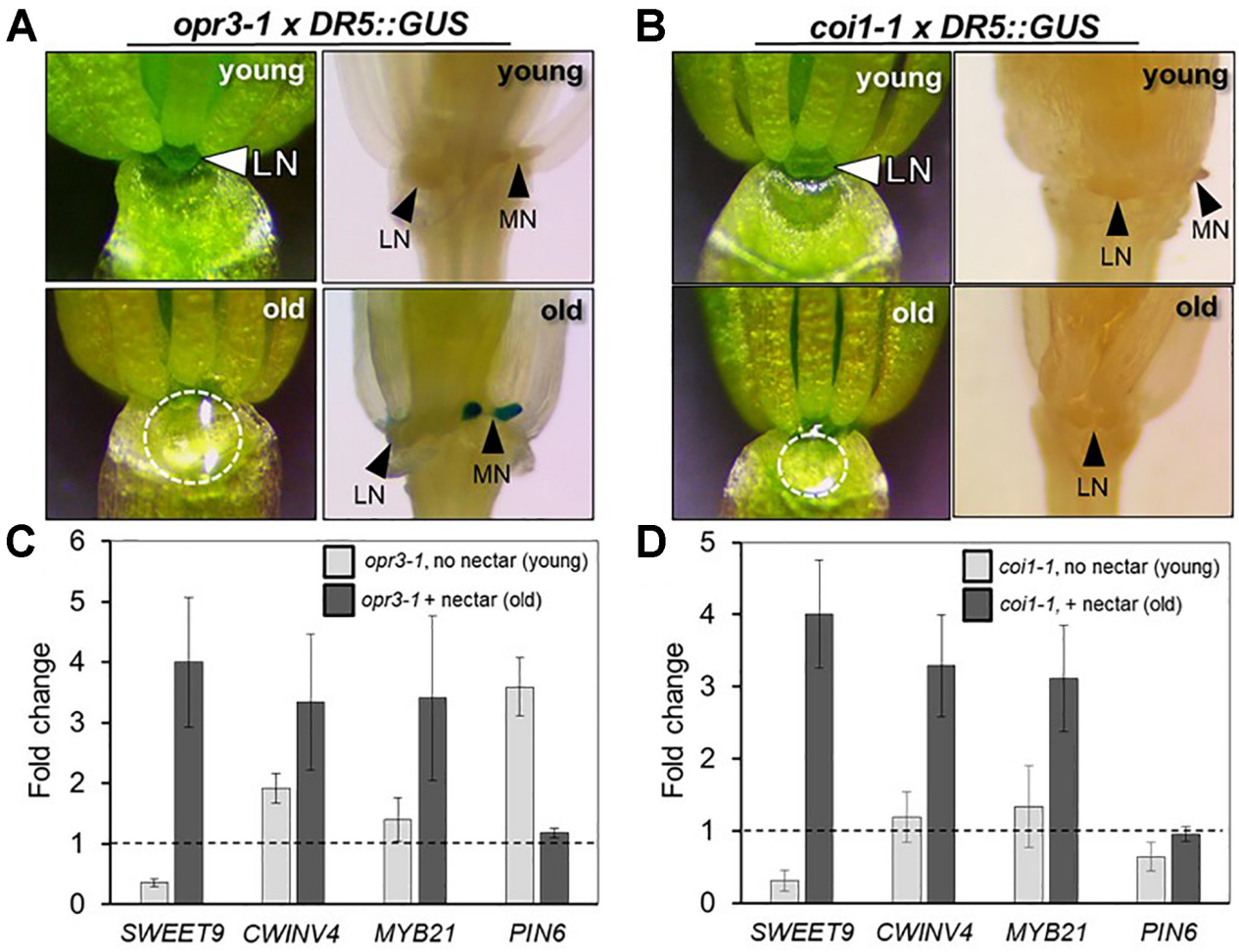

FIGURE 4 | The JA receptor COI1 is not required for nectar secretion, but is required for the nectary auxin response. The 'young' flowers of *opr3-1* **(A)** and *coi1-1* **(B)** do not produce nectar, whereas the corresponding 'old' flowers secrete nectar. 'Old' *opr3-1* flowers (with nectar) display a strong auxin response in nectaries (**A**, lower right), but the corresponding 'old' *coi1-1* flowers with nectar do not (**B**, lower right). The expression of key genes involved in nectar secretion was examined in whole flowers by qRT-PCR in both 'young' (no nectar) and 'old' (with nectar) of *opr3-1* **(C)** and *coi1-1* **(D)**. qRT data was normalized to Col-0 (dashed line).

Starch Metabolism Is Altered in Nectaries of JA Mutants

Starch metabolism has been shown to be important in the process of proper nectary function (Paschold et al., 2008; Ruhlmann et al., 2010; Lin et al., 2014; Wang et al., 2014). Starch buildup is required in nectaries prior to nectar production and is rapidly broken down to produce sucrose and hexoses during the secretory phase. In tobacco, starch metabolism was severely compromised in a *coi1* mutant leading to an absence of nectar production (Wang et al., 2014). We hypothesized that *aos-2,* a JA biosynthetic mutant, would also display defects in starch metabolism. To test this hypothesis, Stage 14 WT and *aos-2* flowers were stained with Lugol's iodine and imaged under a dissecting microscope. The distinct staining of the flower peduncle with iodine suggests a buildup of starch whereas lesser staining is consistent with the breakdown of the starch for nectar production. Also, the stomata on the nectaries stain distinctively when nectar secretion is occurring. Our study correlates a breakdown of starch with nectar secretion in the WT flowers (**Figures 7A,B**) whereas the *aos-2* flower peduncles have increased starch accumulation and a lack of stomatal staining (**Figures 7C,D**), as expected by the nectarless phenotype. The starch staining patterns observed in *aos-2* are very similar to those observed in *cwinv4* and *sweet9* mutants (Ruhlmann et al., 2010; Lin et al., 2014). Furthermore, since *coi1* mutants in tobacco showed defects in nectary starch metabolism and degradation (Wang et al., 2014), we hypothesized there may be starch defects in the Arabidopsis *coi1-1* mutant. In the nectarless 'young' flowers of *coi1-1* we indeed saw strong staining in both the peduncle and the nectary of the flower (**Figures 7E,F**), whereas in the 'old' flowers that do produce nectar, we see some breakdown of the starch in the peduncle and a mobilization of starch products to the guard cells of the nectary stomata (**Figures 7G,H**). This result confirms JA's role in starch breakdown and subsequent nectar production.

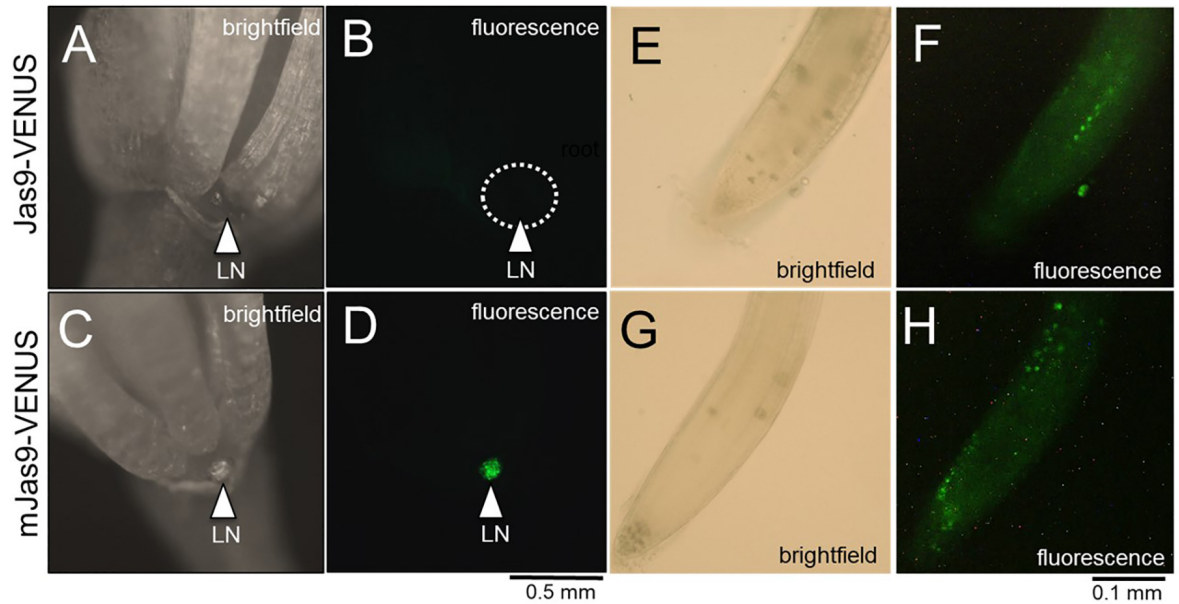

FIGURE 5 | The JA response in mature lateral nectaries. Mature lateral nectaries (Stage 14 flowers with nectar) were evaluated for active JA responses via the Jas9-VENUS and mJas9-VENUS system. Jas9-VENUS is degraded in the presence of active JA signaling (decreased fluorescence), whereas mJas9-VENUS is not degraded (stable fluorescence). **(A)** Jas9::VENUS brightfield and **(B)** corresponding fluorescence in a Stage 14 nectary. **(C)** mJas9::VENUS brightfield and **(D)** corresponding fluorescence image in a Stage 14 nectary. The roots of both Jas9::VENUS **(E,F)** and mJas9::VENUS **(G,H)** display strong VENUS-dependent signal (punctate spots represent nuclei in **G,H**).

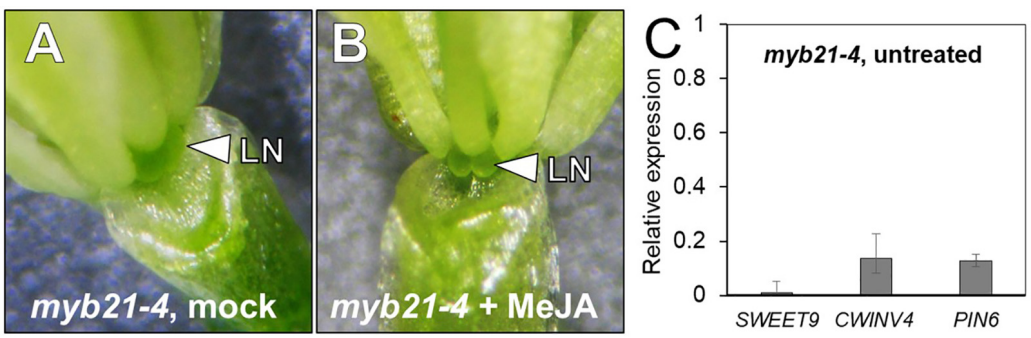

FIGURE 6 | *MYB21* is required for nectar production. The flowers of mock- and MeJA treated *myb21-4* do not produce nectar **(A,B)**. The expression of key genes involved in nectar secretion was examined in whole untreated *myb21-4* flowers by qRT PCR **(C)**. Fold-change was calculated relative to Col-0 (wild-type expression = 1).

DISCUSSION

The Octadecanoic Acid Biosynthetic Pathway Is Required for Nectary Function

Arabidopsis mutants deficient in the octadecanoic pathway did not secrete nectar (**Figures 1–4**), particularly in 'young' open flowers that are the equivalent of Stage 14 wild-type flowers, which actively secrete nectar. Perhaps most telling, *aos-2* did not produce nectar droplets in either 'young' or 'old' flowers, although exogenous MeJA did restore nectar production (**Figures 1**, **2**). *AOS* is a single copy gene indispensable for the jasmonate biosynthesis pathway. Interestingly, the 'old' flowers of *dad1-1* and *opr3-1* did eventually produce nectar, which coincided with the expression of *SWEET9*, a sucrose transporter indispensable for nectar secretion (Lin et al., 2014). These results could possibly be explained by the fact that neither *dad1-1* nor *opr3-1* are completely JA deficient. *DAD1* encodes a phospholipase with extensive functional redundancy, as demonstrated by the fact that *dad1-1* mutants are defective in anther dehiscence and pollen development but are still able to accumulate JA upon wounding via the *DAD1* leaf homolog *DONGLE* (Ishiguro et al., 2001; Hyun et al., 2008). *OPR3* is reportedly essential for JA synthesis, but *opr3-1* has a leaky phenotype when exposed to pathogen attack

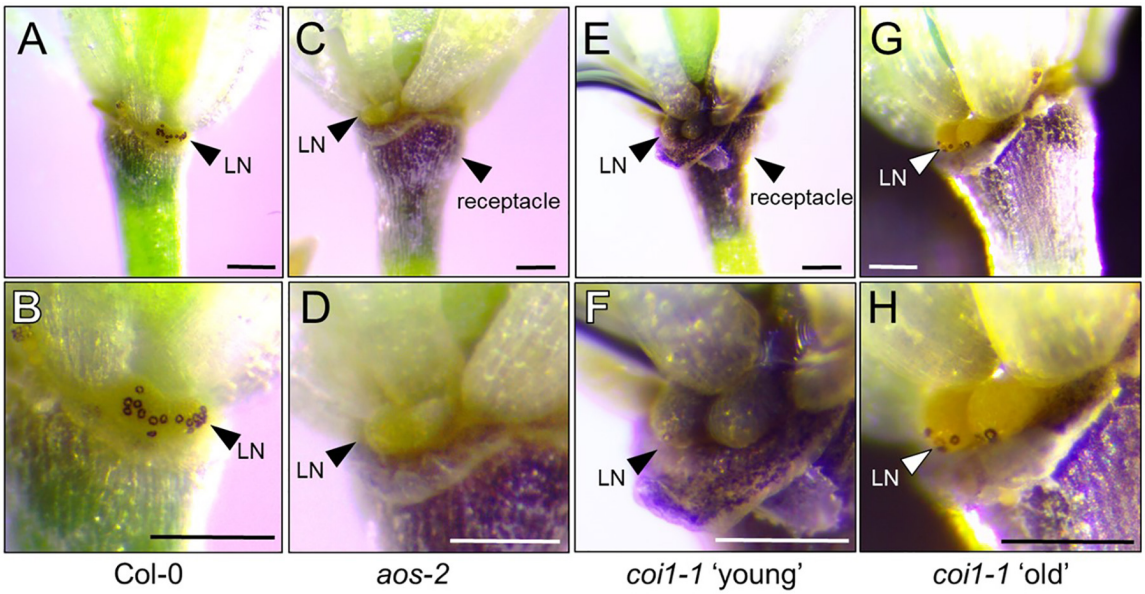

FIGURE 7 | Starch accumulation patterns in *aos-2* and *coi1-1* nectaries. Each pair of images are of the same flower at different magnifications stained for starch accumulation. **(A,B)** Wild-type Col-0; **(C,D)** *aos-2*; **(E,F)** *coi1-1* 'young', **(G,H)** *coi1-1* old. Arrowheads either point out the lateral nectaries (LN) or the receptacle. Note the stained stomates in **(B,H)**. Scale bars = 100 microns.

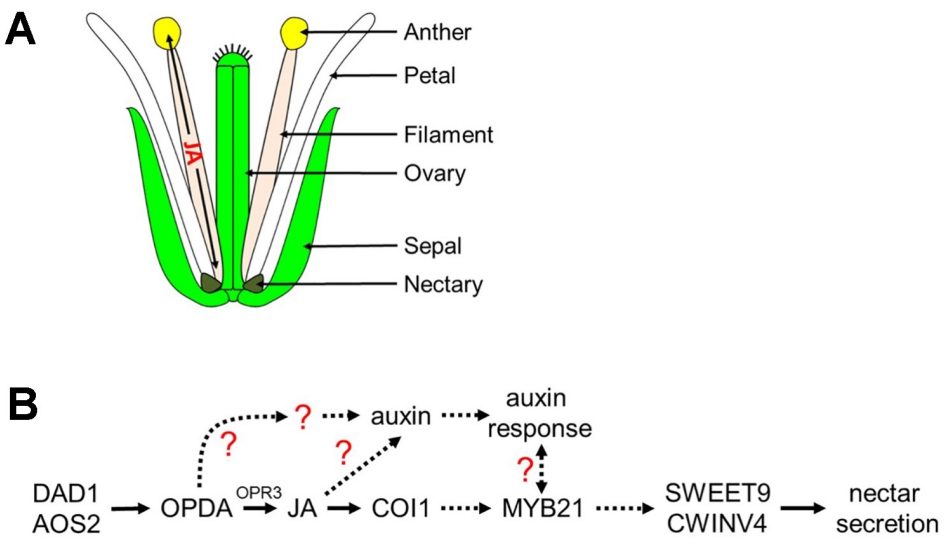

FIGURE 8 | Proposed model of jasmonic acid migration after synthesis in the filament and regulation of nectar secretion. **(A)** JA synthesized in the anther filament migrates down the filament to the nectary to promote nectar secretion, and travels to the anther where it promotes the dehiscence of anthers and pollen maturation. **(B)** JA regulation of nectar secretion. Under standard conditions, JA or its precursors are synthesized in the filament leading to the induction of *MYB21* expression which in turn is required for nectar secretion via the expression of *SWEET9* and *CWINV4*. JA also regulates auxin responses within the nectaries, another hormonal factor involved in nectar secretion. A possible role for a JA- and COI1-dispensible pathway via OPDA for nectar secretion is indicated; dashed arrows represent hypothetical interactions based on limited data.

(Chehab et al., 2011). It was also recently reported that in the absence of OPR3, OPDA could enter the β-oxidation route to produce 4,5-didehydro-JA which can subsequently act as a precursor to JA and JA-Ile (Chini et al., 2018). These results perhaps suggest that JA may still be able to accumulate to sufficient concentrations in *dad1-1* and *opr3-1* to restore the necessary signaling to rescue nectar secretion. However, it is important to note that anther dehiscence was not observed in any of the JA synthesis or response mutants, in either young or old flowers (data not shown). These results suggest that JA is not accumulating to high levels in these mutants and indicate that anther dehiscence is not required for nectar production.

COI1 Is Dispensable for Nectar Secretion in Arabidopsis

The presence of nectar in 'old' *opr3-1* and *coi1-1* flowers (**Figure 4**) could also indicate the presence of an octadecanoic acid-dependent but JA- and COI1-dispensable signaling pathway in relation to nectary function. The substrate for OPR3 is *cis*-OPDA, which accumulates in *opr3-1* instead of being converted to JA (**Supplementary Figure S1**). *cis*-OPDA has been reported to be a potent signaling molecule that can regulate gene expression in either a COI1-dependent or COI-independent fashion (Dave and Graham, 2012). For JA responses to occur, JA-Ile is usually rapidly synthesized in response to an environmental or developmental cue which then binds the F-box protein COI1, which targets JAZ repressors for degradation via the 26S proteasome (Sheard et al., 2010; Pérez and Goossens, 2013; Wasternack and Hause, 2013). This subsequently liberates the transcription factors that drive JA-related gene expression. In the *jar1-11* mutant, where JA-Ile levels are severely compromised (Suza and Staswick, 2008), nectar production proceeds normally in 'young' flowers (**Supplementary Figure S5**). This suggests that the low levels of JA-Ile might suffice for nectar production or that nectar production might not require the well-established COI1-dependent JA regulation pathway in Arabidopsis. This latter supposition further gains support from the observation that nectar production proceeds normally in older flowers of the *coi1-1* loss-of-function mutant. It is remarkable though that younger *coi1-1* flowers produced no nectar even at Stages 13–14 when nectar secretion normally begins, indicating a COI1-dispensible pathway might require a time lag in responding to OPDA- or JA-dependent signaling. This delayed nectar phenotype is perhaps even more interesting in light of the reports that JA-Ile/COI1 signaling in wild tobacco flowers (*Nicotiana attenuata*) is required for nectar production (Stitz et al., 2014). Overall, this discrepancy should be further explored across species to better understand the conserved processes that are essential for nectar regulation.

MYB21 Is the Apparent Ortholog of Tobacco *MYB305*

MYB21 is a JA-responsive transcription factor previously reported to be important for stamen elongation and floral maturation (Reeves et al., 2012). It is also closely related to tobacco *MYB305* (*NtMYB305*) (Ren et al., 2007), which is required for nectar production and the expression of nectary-specific genes (Liu et al., 2009; Liu and Thornburg, 2012). In our study, *MYB21* expression was enhanced in *aos-2* flowers exogenously treated with MeJA, supporting its JA-inducibility (**Figure 2**). *myb21-4* flowers also did not produce nectar and also lacked the expression of genes required for nectar production – AtSWEET9, AtCWINV4, and AtPIN6 all displayed large decreases in expression in *myb21-4* (**Figure 6**). SWEET9 acts as a sucrose transporter, putatively transporting sucrose out of nectary parenchyma cells (Lin et al., 2014). After sucrose export, CWINV4 converts this disaccharide into its hexose monomers, glucose and fructose (Ruhlmann et al., 2010). Null mutants for these genes phenocopy one another as neither produce nectar, while maintaining normal nectary structure, and have heavy starch accumulation in the floral receptacle (Ruhlmann et al., 2010; Lin et al., 2014). The regulation of these nectar sugar transporters and invertases is vital for proper nectar production and secretion. Thus, MYB21 appears to regulate both *CWINV4* and *SWEET9* expression, in addition to *PIN6*, but it is currently unknown if MYB21 directly or indirectly controls their expression. It was interesting to note the expression of *SWEET9* was strongly reduced in the younger flowers of JA mutants such as *aos-2*, *opr3-1,* and *coi1-1* but gradually increased in the older flowers or in response to endogenous and exogenous auxin. These results suggest that SWEET9 expression proceeds via a COI1-dispensable, but MYB21-dependent route (**Figure 8**).

Crosstalk Between JA and Auxin Pathways

There are a number of well-known interactions between auxin (IAA) and JA in plants, both in terms of homeostasis and downstream response (Pérez and Goossens, 2013; Wasternack and Hause, 2013). One of the key discoveries of our study was a further elucidation for the role of JA during nectar production and how it coordinates with auxin responses. Our study reveals that treatment of flowers with exogenous synthetic auxin (1-NAA) can rescue nectar production defects in JA biosynthetic mutants such as *aos-2* and *dad1-1*, which fail to produce nectar in 'young' open flowers (**Figure 2**). We also found that auxin responses in the nectaries of the 'young' open flowers of *dad1-1, aos-2* and *opr3-1* were absent as revealed by the DR5::GUS auxin reporter system. This suggests that the octadecanoic acid biosynthetic pathway is required for nectary auxin responses and subsequent nectar production. Auxin responses reappeared in *aos-2* flowers treated with MeJA suggesting that jasmonates can increase auxin biosynthesis in the nectaries. It remains to be tested whether free auxin levels are indeed diminished in the mutant flowers and whether they do increase after the MeJA treatment. However, we did demonstrate that nectary-derived auxin biosynthesis (via SWEET9pro::*iaa*M) can rescue nectar production in the *aos-2* background, likely by inducing SWEET9 expression (**Figure 3**). Cumulatively, these results indicate that auxin acts downstream of JA to induce nectar secretion.

The complete absence of an auxin response in *coi1-1* nectaries, even after nectar production occurred in older flowers, is particularly interesting (**Figure 4**). The possibility that nectar production occurs independently of COI1 in Arabidopsis has been discussed previously. This suggests that nectar production and a canonical auxin response via the TIR1 pathway in the nectary are separable events. Both auxin and JA use a similar mechanism of signaling by forming specialized co-receptor complexes known as SKP1-Cullin-F-box protein (SCF) E3 ubiquitin ligase complexes. TIR1 and COI1 act as the F-box proteins providing specificity for auxin and JA in these complexes, respectively. This complex subsequently binds their target proteins, Aux/IAA (auxin) and JAZ (JA), respectively,

which are then degraded via the ubiquitin-proteasome system (Kelley and Estelle, 2012) thus allowing hormone specific transcriptional activation to proceed. Both these hormones are perceived by shared components of a SCF-E3 ligase system, and it has been demonstrated that disruption in the complexes can cause an impairment of hormonal responses (del Pozo et al., 2002; Quint et al., 2005; Moon et al., 2007; Pérez and Goossens, 2013). Thus, there is a possibility that the loss of COI1 disrupts the equilibrium of the shared aspects of the ubiquitin-proteasome system thus affecting auxin signaling and responses too.

An Alternative Mechanism of JA Involvement in Nectar Production?

We should also consider alternative routes of how jasmonates and related oxylipins might affect nectar production. A potential for JA regulation of nectar secretion via control of water movement also exists. Proper timing of pollen release and nectar secretion in flowering plants can be vital for reproduction, therefore, there may be a close singular upstream mechanism between anther dehiscence and nectar secretion. Baum et al. (2001) characterized many male sterile mutants to be deficient in nectar production. This same correlation between anther dehiscence/male-sterility and nectar production was observed in different male sterile mutants of our study. Ishiguro et al. (2001) determined that water transport was halted in the vascular tissue of *dad1-1* anthers and suggested that JA regulates water transport in the male organs. Later, Ruhlmann et al. (2010) suggested that the high amount of nectar sugars leads to the flow of water out of the nectary, creating the nectar droplet presented at the base of the sepal. Perhaps water transport in the nectaries is disrupted by the lack of JA which leads to the absence of nectar in the JA synthesis and response mutants. Therefore, only when the proper JA response can be restored in the JA synthesis mutants can the water transport/nectar secretion be restored as well. However, the molecular mechanism by which water flow is disrupted to *dad1-1* stamens, and perhaps nectaries, is currently unknown.

Cumulatively, the results from this study implicate the octadecanoic acid biosynthetic pathway and auxin as indispensable regulators of nectar secretion independent of *COI1* in Arabidopsis. We propose a model (**Figure 8**) in which *cis*-OPDA may act as a signaling molecule upstream of *MYB21* and auxin responses leading to the expression of *SWEET9* and *CWINV4*. Still, certain aspects of the proposed model are unclear, particularly the connection between the auxin response and *MYB21* (dashed line with question mark), as exogenous treatment of *aos-2* flowers with NAA induced *SWEET9* expression and nectar secretion while concurrently downregulating *MYB21* expression (**Figure 2**). A possible explanation for the apparent discrepancy between *MYB21* and *SWEET9* expression in auxin-treated *aos-2* flowers is that qRT-PCR analyses were done with whole flowers (due to the extremely small size of Arabidopsis nectaries) and *MYB21* is not a nectary-specific gene (Reeves et al., 2012), unlike *SWEET9* and *CWINV4* (Ruhlmann et al., 2010; Lin et al., 2014). Therefore, it is possible that *MYB21* expression might be downregulated in non-nectary tissues of NAA-treated *aos-2* flowers, while actually being upregulated in nectaries. Further studies will focus on examining this crosstalk and IAA, JA, and *MYB21* in nectar secretion, as well as their respective control of *CWINV4* and *SWEET9* expression.

AUTHOR CONTRIBUTIONS

AS, RR, PK, MJ, and CC designed and executed the experiments. AS, RR and CC wrote the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01060/full#supplementary-material

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Nectar-Secreting and Nectarless *Epidendrum*: Structure of the Inner Floral Spur

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Epidendrum, the largest genus of Neotropical orchids, contains both nectar-secreting and nectarless species. Here, we compare the fine structure of the inner floral spur, termed the cuniculus, in nectariferous (*E. difforme*, *E. nocturnum, E. porpax, E. rigidum, E. vesicatum*) and seemingly nectarless (*E. capricornu*, *E. ciliare, E. criniferum, E. pseudepidendrum*, *E. radicans, E. xanthoianthinum*) species. This is the first time for such a detailed investigation of cuniculus structure to be undertaken for *Epidendrum.* Our aim was to characterize features indicative of secretory activity and to ascertain whether flowers presumed to be nectarless produce alternative pollinator food-rewards. The cuniculus is formed by fusion of the basal part of the labellum and column and extends alongside the ovary and transmitting tract. Our study indicates that all investigated species produce nectar or nectar-like secretion to varying degrees, and no alternative pollinator food-rewards were observed. Even though macroscopic investigation of presumed rewardless species failed to reveal the presence of secretion within the cuniculus, close observations of the cells lining the cuniculus by LM, SEM, and TEM revealed the presence of cuticular blisters and surface material. Moreover, the similarity of both the thick tangential cell walls (with the exception of *E. vesicatum*) and organelle complement of cuniculus epidermal cells in both copiously nectariferous species and those producing only small quantities of surface secretion confirmed the presence of secretory activity in species generally regarded to be rewardless. The secretory character was particularly obvious in the cells of the cuniculus of *E. nocturnum,* but also in *E. ciliare, E. radicans* and *E. xanthoianthinum,* since electron-dense cytoplasm and mitochondria, ER and secretory vesicles were abundant. Furthermore, cell wall protuberances occurred in *E. nocturnum*, which was indicative of intense transmembrane transport. This investigation highlights the need to examine more closely whether *Epidendrum* spp. considered to lack food-rewards based solely on macroscopic examination really are rewardless and deceptive.

Keywords: *Epidendrum*, Orchidaceae, nectar, nectary, secretory tissues, floral rewards, cuniculus

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INTRODUCTION

Orchids offer their pollinators a variety of floral food-rewards, such as nectar, oil and edible trichomes, with many more producing non-food rewards, such as fragrances, waxes and resins. Based on analyses by Neiland and Wilcock (1998), the presence of nectar in both temperate and tropical orchids can increase their reproductive success (fruit set). In Orchidaceae, nectar is the most common floral food-reward, and here, perigonal nectaries located on the labellum predominate (Bernardello, 2007; Davies and Stpiczyńska, 2008). They may occur in shallow depressions, as in *Epipactis* (Pais, 1987; Kowalkowska et al., 2015), on the labellar callus, as in *Maxillaria anceps* (Davies et al., 2005), in the median furrow of the labellum, as in *Listera* (van der Cingel, 2001) and *Bulbophyllum* (Stpiczyńska et al., 2015, 2018), in the labellum base, as in *Cleistes* (Pansarin et al., 2012), *Elleanthus* (Nunes et al., 2013) and *Psilochilus* (Pansarin and Amaral, 2008a), but also on the column, as in *Maxillaria coccinea* and *Ornithidium sophronitis* (Stpiczyńska et al., 2004, 2009), or in the mentum, as in *Dendrobium finisterrae* (Kamińska and Stpiczyńska, 2011). However, the most frequently encountered type of nectary, occurring both in this enormous family and also in other angiosperms, is the nectary spur, which is present in at least 0.60% of angiosperm genera (Mack, 2013; Mack and Davis, 2015). Nectary spurs of various lengths occur as outgrowths of the labellum in representatives of Aeridiinae (Davies and Stpiczyńska, 2008; Stpiczyńska et al., 2011), Maxillariinae (Davies and Stpiczyńska, 2007), Orchidinae (Stpiczyńska, 2003; Bell et al., 2009), and Spiranthinae (e.g., Pansarin and Ferreira, 2015). In *Anacamptis pyramidalis* f. *fumeauxiana* (Orchidinae), in addition to the spur formed at the base of the labellum, two spurs originating from lateral sepals are present (Kowalkowska et al., 2012). In Laeliinae, the nectary, if present, is represented in the majority of cases by a cuniculus – an atypical inner spur formed by fusion of the column and labellum throughout their length, and which runs deep alongside the transmitting tract and ovary.

Regardless of taxonomic position and the presence or absence of floral rewards, the spurs in Orchidaceae studied to date were lined by flat epidermal cells (e.g., *Schoenorchis gemmata* – Stpiczyńska et al., 2011), or conversely, the epidermis was papillose (e.g., *Ascocentrum*) or trichomatous (e.g., *Angraecum germinyanum*, *Papilionanthe vandarum, Platanthera, Dactylorhiza, Brassavola*) (Stpiczyńska, 2003; Davies and Stpiczyńska, 2008; Bell et al., 2009; Stpiczyńska et al., 2010, 2011, respectively). Beneath the secretory epidermis occurred one to several layers of small subepidermal parenchyma cells. Published, detailed, microscopical analyses revealed diverse sculpturing and variable thickness in the cuticle overlying the secretory epidermal cells. Cuticular blisters were observed in *Platanthera* (Stpiczyńska, 2003) and *Schoenorchis gemmata* (Stpiczyńska et al., 2011), but pores were rarely recorded (e.g., *Brassavola flagellaris* – Stpiczyńska et al., 2010). Cell walls were predominantly thin or of moderate thickness, with the exception of ornithophilous *Ascocentrum curvifolium* (Stpiczyńska et al., 2011) and moth-pollinated *Brassavola flagellaris* (Stpiczyńska et al., 2010). The cells were interconnected by numerous plasmodesmata. Generally, the ultrastructure of secretory cells of the spur conformed with that of typical nectary cells (Nepi, 2007). These cells contained dense cytoplasm with numerous mitochondria, ER profiles, dictyosomes and secretory vesicles (Stpiczyńska, 2003; Davies and Stpiczyńska, 2008; Stpiczyńska et al., 2010, 2011). Additionally, they often contained plastids with prominent starch grains (e.g., *Ascocentrum* – Stpiczyńska et al., 2011), or were completely starchless throughout the lifespan of the flower (e.g., *Gymnadenia* – Stpiczyńska and Matusiewicz, 2001). In *Papilionanthe vandarum,* starchless plastids contained large deposits of phenolic-like material (Stpiczyńska et al., 2011).

Epidendrum L. is the largest genus of tribe Epidendreae, subtribe Laeliinae, and according to the APG IV website (Stevens, 2001 onward), comprises 1425 species. It is distributed from the southeastern United States of America to northern Argentina (Hágsater and Soto-Arenas, 2005). It comprises both food-rewarding and food-deceptive species (Pansarin, 2003; Pansarin and Amaral, 2008b; Pansarin and Pansarin, 2014, 2017). Although its flowers are visited by a wide range of pollinators, moths and butterflies are the most frequently recorded, and according to Pinheiro and Cozzolino (2013), this kind of specialization (together with certain novel vegetative characters) may represent key innovations that led to the enormous degree of speciation found in this genus. Ornithophily has been reported for *E. cinnabarinum*, *E. ibaguense,* and *E. pseudepidendrum* (van der Pijl and Dodson, 1969; van der Cingel, 2001). Orange-red or yellow flowers are attributed to butterfly-pollinated species, whereas whitish to pale green, highly fragrant flowers are predominantly moth-pollinated (van der Pijl and Dodson, 1969; van der Cingel, 2001). In some moth-pollinated species, scent is produced by osmophores (Pansarin and Pansarin, 2017). *Epidendrum densiflorum* (= *E. paniculatum*) is pollinated by both butterflies and Arctiidae moths (Pansarin, 2003), whereas *E. avicula*, is pollinated by several species of micro-moths, as well as Tipulidae or crane flies (Pansarin and Pansarin, 2017). In fact, many *Epidendrum* species have a long cuniculus and are thus adapted for pollination by Lepidoptera (Pansarin, 2003; Pansarin and Amaral, 2008b; Fuhro et al., 2010; Pansarin and Pansarin, 2017). Conversely, although other members of Laeliinae have also long been considered to possess a cuniculus (e.g., Dressler, 1993), some taxa, such as *Amblostoma* and *Lanium*, both currently included in *Epidendrum sensu lato*, lack this character (Pansarin and Pansarin, 2014, 2017). Study of the reproductive biology of *E. tridactylum*, a member of the *Amblostoma* group, demonstrated that this species produces fragrant and rewardless flowers, and attracts dipterans that drink the extra-floral nectar produced at the base of the floral bracts (Pansarin and Pansarin, 2014). As in *E. tridactylum*, the flowers of *E. avicula* lack a cuniculus, and the nectary is located at the base of the labellum, inside a tube formed by the labellum and column. As a consequence, insects possessing a short but thin proboscis (i.e., flies and micro-moths) are the most effective pollinators of these orchids (Pansarin and Pansarin, 2017).

In the majority of *Epidendrum* spp., insects searching for nectar insert their proboscides into the cuniculus. Since the entrance to the cuniculus has a keyhole-like structure, such behavior causes the pollinator to become temporarily detained. The traumatized insect thus avoids revisiting the same inflorescence, thereby reducing geitonogamy, or pollen loss in the case of self-incompatible species (Dressler, 1981; Pansarin and Pansarin, 2017). As in many other orchids, flowers of *Epidendrum* are infrequently visited, and low fruit set is common (Adams and Goss, 1976; Ackerman and Montalvo, 1990; Almeida and Figueiredo, 2003; Pansarin and Amaral, 2008b; Fuhro et al., 2010; Pinheiro et al., 2010, 2011).

Despite the presence of a cuniculus, nectar has only rarely been found in *Epidendrum*, and to date, its presence has been recorded only for *E. difforme* (Goss, 1977), *E. compressum*, *E. schlechterianum*, *E. strobiliferum* (Braga, 1977) and *E. avicula* (Pansarin and Pansarin, 2017).

It should be emphasized that reward-producing and rewardless *Epidendrum* species have so far mainly been distinguished by macroscopic observation for the presence or absence of nectar within the inner spur (Almeida and Figueiredo, 2003; Hágsater and Soto-Arenas, 2005; Pansarin and Amaral, 2008b). Detailed structural studies of the cuniculus are scarce, particularly in species where nectar appears to be absent. This is the first time for such a detailed investigation of cuniculus structure to be undertaken for *Epidendrum.* For this study, we selected 11 species of *Epidendrum* that differ in their type of pollination syndrome. The aim of this research is to: (i) compare the structure of the cuniculus in nectariferous species of *Epidendrum* and those regarded to be nectarless; (ii) explore whether the presence of nectar and the structure of the cuniculus are correlated; (iii) check whether flowers assumed to be nectarless produce alternative pollinator rewards.

MATERIALS AND METHODS

The majority of plants used in this study were grown at the Botanic Garden of the University of Warsaw, Poland. They include nectar-secreting *Epidendrum difforme* Jacq., *E. nocturnum* Jacq., *E. porpax* Rchb. f., *E. rigidum* Jacq., and seemingly nectarless *E. capricornu* Kraenzl., *E. ciliare* L., *E. criniferum* Rchb. f., *E. pseudepidendrum* Rchb. f., *E. radicans* Pav. ex Lindl. and *E. xanthoianthinum* Hágsater. The sole exception was the nectar-secreting *E. vesicatum* Lindl. which was collected in the city of Blumenau, state of Santa Catarina, South Brazil and cultivated at the LBMBP Orchid House, University of São Paulo, Ribeirão Preto, Brazil. The species cultivated at the Botanic Garden of the University of Warsaw were grown in a glasshouse at 25°C, and those which flowered in autumn/winter (*Epidendrum capricornu, E. ciliare E. difforme, E. nocturnum, E. porpax, E. rigidum*) were provided with a photoperiod comprising 12 h light and 12 h darkness. AGRO, PILA, MT WLS400W-Z-00 lamps were used to supplement light during the day. The study was conducted on 1-2 plants of each species, and 5 flowers each were used for microscopical analysis. Abbreviations of authorities for plant names follow Brummitt and Powell (1992) throughout.

The position of the cuniculus and the presence of nectar in longitudinally sectioned flowers on the first day of anthesis were determined by means of a Nikon SMZ100 stereomicroscope. The structure of the tissues surrounding the cuniculus was subsequently examined using light microscopy (LM), including fluorescence microscopy (FM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The number of vascular bundles supplying the tissues surrounding the cuniculus was recorded based on transverse sections of the flower taken at the level of insertion of the perianth segments. We considered vascular bundles present in parenchyma surrounding the cuniculus, but not those located near the transmitting tract.

For microscopical observations, pieces of ovary, together with the cuniculus, were excised and fixed in 2.5% (v/v) glutaraldehyde/4% (v/v) formaldehyde in phosphate buffer (pH 7.4; 0.1 M) for 2 h at 4°C, washed three times in phosphate buffer and post-fixed in 1.5% (w/v) osmium tetroxide solution for 1.5 h at 0°C. The fixed material was then dehydrated using a graded ethanol series, and infiltrated and embedded in LR White resin (LR White acrylic resin, medium grade, Sigma). Following polymerization at 60°C, sections were cut at 70 nm for TEM using a Reichert Ultracut-S ultramicrotome and a glass or diamond knife, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined using a FEI Tecnai Spirit G2 transmission electron microscope, at an accelerating voltage of 90 kV.

Semi-thin sections (0.9–1.0 μm thick) were prepared for LM and FM. For general histology, they were stained with a 1:1 solution of 1% (w/v) aqueous methylene blue: 1% (w/v) aqueous azure II (MB/AII) for 5–7 min.

Histochemical tests were used to detect the presence of lipids and starch in the tissues by treating them with a saturated ethanolic solution of Sudan III and with IKI solution, respectively, followed by examination using a Nikon E-200 or Nikon Eclipse 90i light microscope. The periodic acid-Schiff (PAS) reaction was also employed to detect the presence of insoluble polysaccharides (Jensen, 1962). Semi-thin sections were also treated with auramine O (Gahan, 1984) and examined using FM with FITC filter (excitation light 465–495 nm, barrier filter 515–555 nm) to detect the presence of lipid. A UV2B filter (Nikon) was used to check for chlorophyll autofluorescence. Micrometry and photomicrography were accomplished by means of a Nikon Eclipse 90i (NIS-Elements AR software) or a Stereozoom Leica S8 APO stereomicroscope, in conjunction with a PC employing IM50 image analysis software. For TEM images, the FEI Tecnai Spirit G2 TEM Imaging & Analysis computer program was used. Thicknesses of cell wall and cuticle were measured only for species on which TEM analysis was performed, and the mean calculated (n = 10 measurements ± SD).

For SEM, fixed pieces of the flower, cut longitudinally to expose the cuniculus, were dehydrated and subjected to critical-point drying using liquid CO_2. They were then sputter-coated with gold and examined using a Vega II LS scanning electron microscope at an accelerating voltage of 10 kV.

RESULTS

Species With Nectar Visible Upon Macroscopic Observation

The cuniculus of the light-green flowers of *Epidendrum difforme* was 10 mm long and contained nectar. The entire inner surface of the cuniculus was coated with nectar. A droplet of nectar was also visible on the adaxial surface of the labellum (**Figure 1A**). The flowers did not produce perceptible fragrance. Epidermal cells lining the cuniculus were flat along the whole length of the cuniculus, with coarse cuticular ridges (**Figures 1B–E,G**). Large deposits of secreted material were present on their surface (**Figure 1B**). Transverse sections revealed the thick (7.42 μm ± 1.44), lamellate, cellulosic walls of epidermal cells (**Figures 1E–G**), and the irregular outline of the outer tangential wall. This was due to numerous wall protuberances. The overlying cuticle was 1.02 μm ± 0.12 thick (**Figures 1D,E,G**). Deposits of electron-translucent material were present beneath distensions of the cuticle, and similar material also occurred on the surface of the epidermis (**Figures 1D,G**). The underlying, single-layered, secretory parenchyma had only slightly thickened tangential walls. Protoplasts of epidermal cells were electron dense (**Figure 1F**) and these, in semi-thin sections, stained intensely with MB/AII (**Figure 1E**). Protoplasts of subepidermal parenchyma were also electron dense, but contained relatively large vacuoles. Typical ground parenchyma cells with thin cell walls, a thin layer of parietal cytoplasm, and a large vacuole, occurred ventral to the cuniculus. Plastids in epidermal, subepidermal and ground parenchyma cells only occasionally contained minute starch grains. However, they contained numerous electron-dense globules. Collateral vascular bundles (three main and tree smaller bundles located alternately) embedded in the ground parenchyma did not penetrate the secretory tissue. Parenchyma cells contained intravacuolar deposits of phenolic-like material (**Figures 1C–E**).

In *Epidendrum nocturnum*, the flowers are greenish-white and fragrant. The cuniculus was 46 mm long and contained copious nectar (**Figures 2A,B**). The epidermis enclosing the cuniculus was composed of small, slightly convex cells (**Figures 2C–G**). The hypodermal cells were also small, and beneath these occurred typical ground parenchyma supplied by three main collateral vascular bundles and several phloem strands (**Figure 2G**). Epidermal and hypodermal cells possessed thick (9.91 μm ± 7.13), cellulosic, lamellate, tangential walls (**Figures 2E–I**). Numerous protuberances projected from the cell walls (**Figure 2I**). The cuticle overlying the epidermis was relatively thin (0.60 μm ± 0.21), as seen in transverse section (**Figure 2F**), and bilayered, the outer layer being lamellate and electron dense (**Figure 2H**). Coarse cuticular ridges and distensions were visible using SEM, and secretory residues were present on the surface of the cuticle (**Figure 2C**). Epidermal and subepidermal parenchyma cells contained dense cytoplasm and large nuclei (**Figures 2E,H–J**). Dictyosomes, mitochondria, ER profiles and numerous secretory vesicles were present in the cytoplasm (**Figures 2I,J**). The plasmalemma was invaginated, and the periplasmic space contained secretory material (**Figure 2I**) or secretory vesicles. In epidermal cells, plastids contained only minute starch grains (**Figures 2I,J**) that were not detectable with the PAS reaction, but starch was more abundant in the ground parenchyma adjacent to vascular bundles (**Figure 2G**). Chloroplasts occurred exclusively in ground parenchyma cells.

The cuniculus of the small, non-fragrant, brown-green flowers of *Epidendrum porpax* was 6 mm long. It had a relatively wide entrance, but tapered toward its base (**Figures 3A,B**). Minute droplets of nectar were visible on the inner surface of the cuniculus using a stereomicroscope, and nectar residues were visible on the cuticle surface using SEM and LM (**Figures 3C–F**). Epidermal cells lining the cuniculus were smaller than those of the hypodermis, and only the outer tangential walls of the epidermal cells were thickened (**Figures 3D,H**). The cuticle overlying the epidermis was thin, ridged, and occasionally distended (**Figure 3E**). Starch was absent from the epidermis and subepidermal parenchyma, but present in ground parenchyma cells (**Figure 3F**), whereas chloroplasts occurred in the subepidermal parenchyma cells (**Figure 3G**). Both epidermal and parenchyma cells contained intravacuolar phenolic-like compounds (**Figure 3H**). Three collateral vascular bundles ran through the ground parenchyma.

In the small, non-fragrant, green flowers of *Epidendrum rigidum*, the cuniculus was 8 mm long, with a narrow entrance, expanding basally (**Figure 4A**), and containing a small volume of nectar. The cells lining the cuniculus were flat or slightly convex (**Figures 4B–G**), thick-walled (5.97 μm ± 1.30), and had a thick (1.75 μm ± 0.47), intact cuticle. Secreted residues were visible on the cuticle using LM, SEM and TEM (**Figures 4C,E,G,H**). Both inner and outer tangential walls of the small epidermal cells, and those of 1-2 layers of the larger subepidermal cells, were thickened (**Figures 4B,E,F**) and lamellate (**Figures 4G,H**), the tissues closely resembling lamellar collenchyma. Cavities present in the middle lamellae of epidermal cells contained similar electron-dense material to that deposited on the surface of the cuticle (**Figures 4G,H**). Epidermal and subepidermal cells were similar in structure in that they both contained a centrally located vacuole and parietal cytoplasm, together with a large nucleus, and small plastids with osmiophilic, electron-dense globules (**Figures 4G,H**). Mitochondria and ER arrays were abundant in parietal cytoplasm, and secretory vesicles fused with the plasmalemma. The cells were interconnected by means of numerous primary pit-fields containing plasmodesmata (**Figure 4G**), and such connections were also present between epidermal, subepidermal, and ground parenchyma cells. Through the parenchyma ran three vascular bundles (**Figure 4D**). Starch was present in the ground parenchyma (**Figure 4F**), and chloroplasts were present in the hypodermis and ground parenchyma.

The cuniculus of the greenish-white flowers of *Epidendrum vesicatum* measured ca. 10 mm in length (**Figures 5A,B**). The flowers produced a pleasant fragrance at night. The lumen of the cuniculus was oval in transverse section and tissues were translucent. The flower produced copious amounts of nectar which, owing to the transparency of the tissues, could easily be observed (**Figure 5C**). Secretory tissue was dorsally located

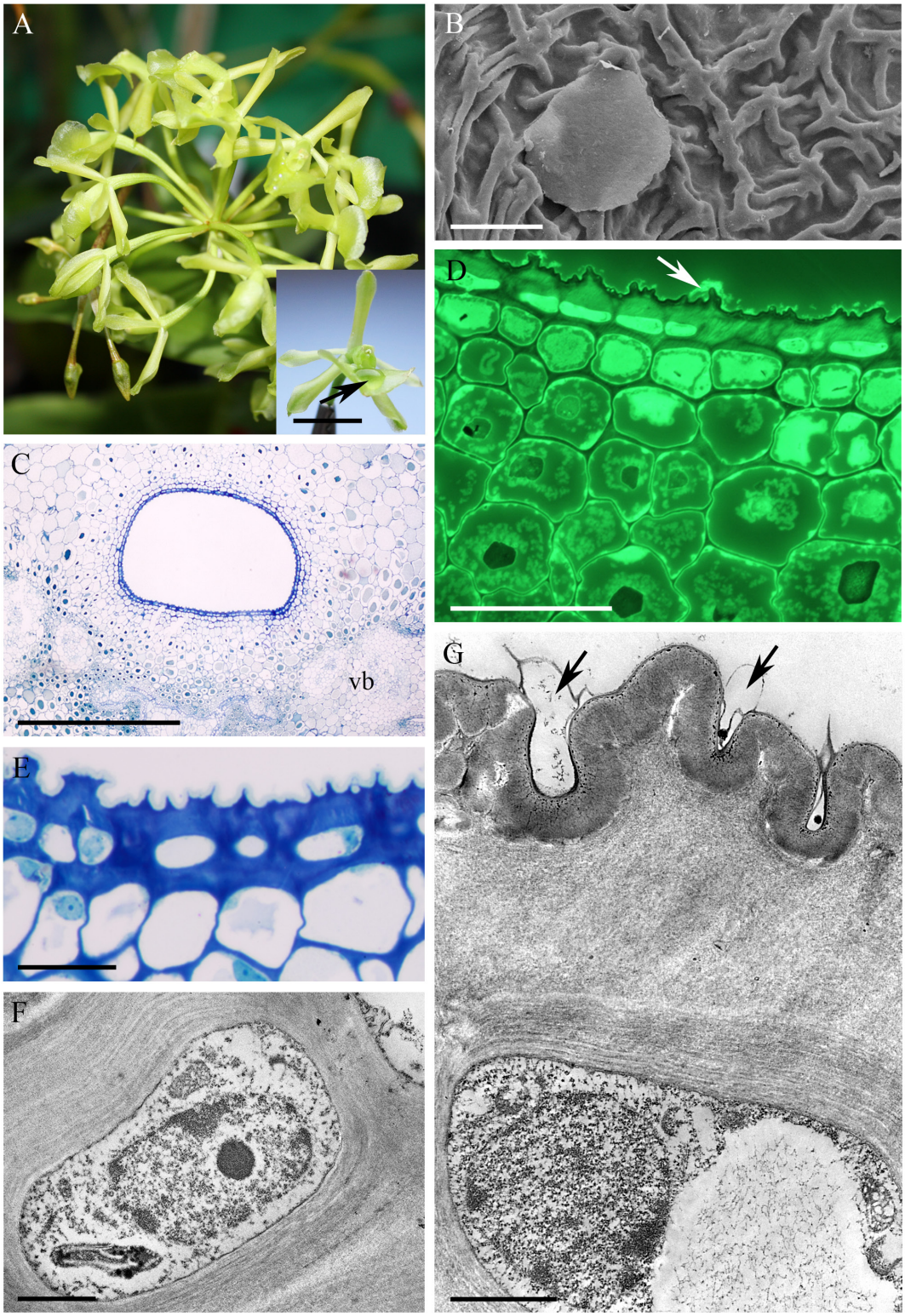

FIGURE 1 | *Epidendrum difforme*. **(A)** Inflorescence. Insert shows flower with droplet of nectar (arrow). **(B)** Convoluted cuticle on surface of epidermal cells with nectar residue. **(C)** Transverse section through cuniculus showing small epidermal cells enclosing the lumen, and parenchyma cells with intravacuolar phenolic-like contents (MB/AII). **(D)** Residues of nectar (arrow) on surface of cuticle. Note thick epidermal cell walls and intravacuolar material (auramine O). **(E)** Detail of epidermis and subepidermal parenchyma (MB/AII). **(F)** Protoplast of epidermal cell. Note the large nucleus and starchless plastid. **(G)** Detail of cell wall and cuticle with associated surface secretion (arrows) of epidermal cells lining the cuniculus. Scale bars: A = 1 cm; B,E = 20 μm; C = 500 μm; D = 50 μm; F,G = 2 μm.

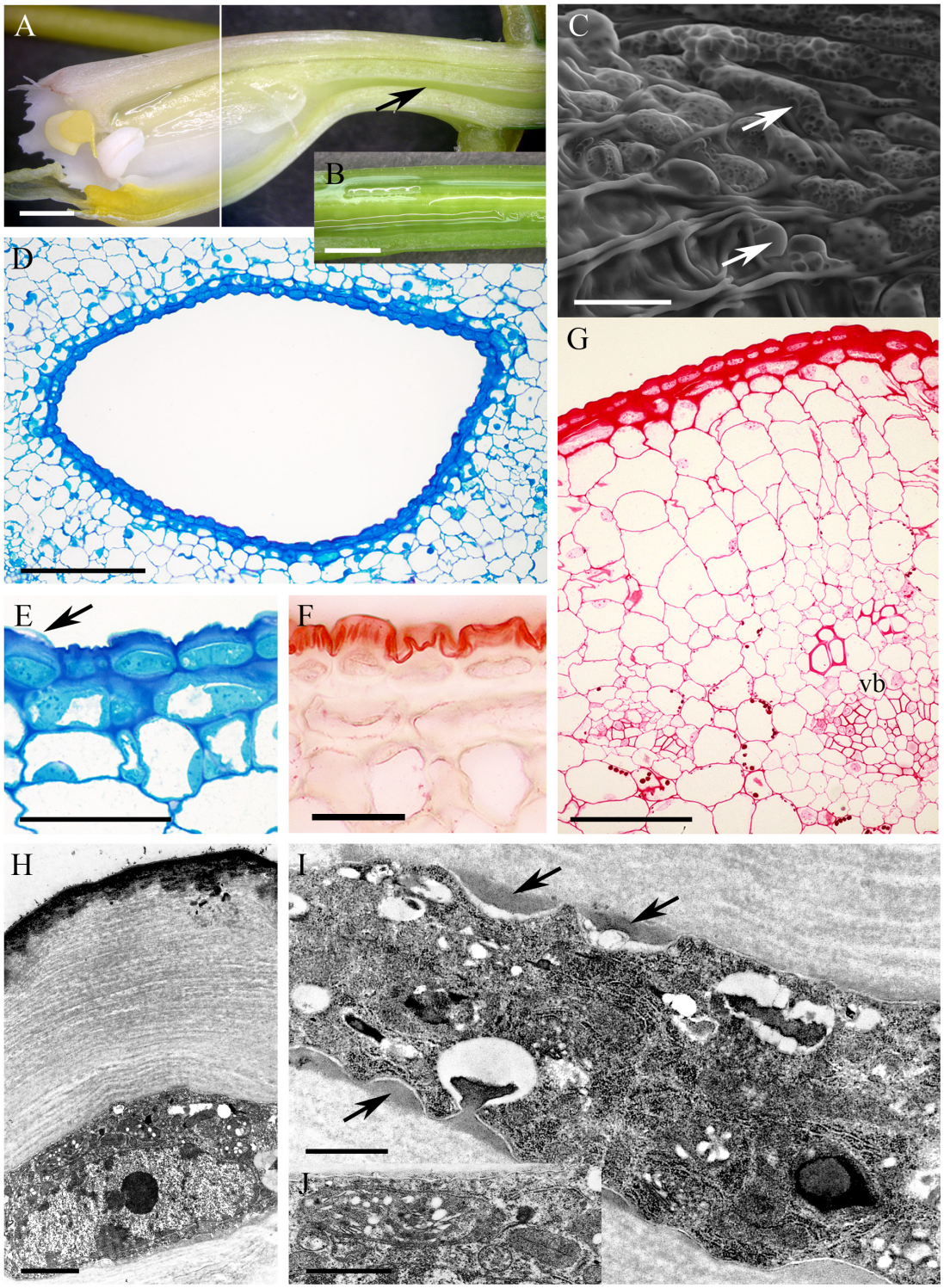

FIGURE 2 | *Epidendrum nocturnum*. **(A)** Longitudinal section of anterior part of the flower showing cuniculus (arrow). **(B)** Detail of cuniculus with copious nectar. **(C)** Epidermal cells with cuticle ridges and cuticular blisters with secretion (arrows). **(D)** Transverse section showing epidermis and subepidermal parenchyma of cuniculus (MB/AII). **(E)** Detail of thick-walled epidermal cells with thin cuticle. Arrow indicates cuticular blister and nectar residues (MB/AII). **(F)** Cuticle lining cuniculus stained with Sudan III. **(G)** PAS reaction stains thick walls of epidermis; large starch grains are located close to vascular bundles. **(H)** Detail of thick outer cell wall and thin cuticle. Note dense protoplast of epidermal cell with large nucleus and plastids. **(I)** Protuberances (arrows) of thick cell wall of epidermal cell. The electron-dense cytoplasm contains numerous ER profiles and plastids. **(J)** Detail of cytoplasm of epidermal cell with plastid containing minute starch grains. A,B = 2 mm; C, E,F = 50 μm; D = 200 μm; G = 100 μm; H = 2 μm; I,J = 1 μm.

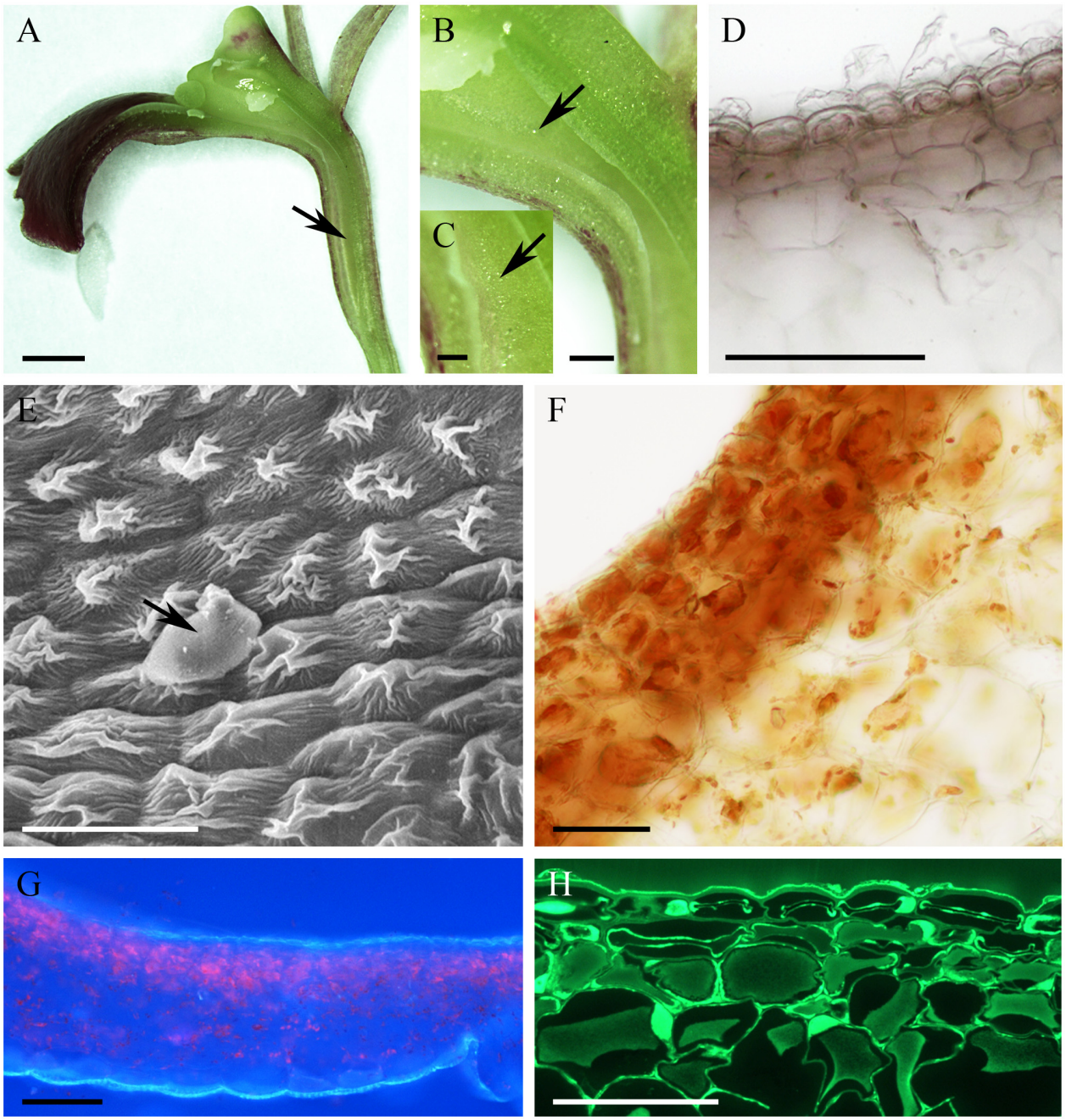

FIGURE 3 | *Epidendrum porpax*. **(A)** Longitudinal section of flower. Cuniculus indicated by arrow. **(B,C)** Details of cuniculus with small droplets of nectar (arrows). **(D)** Epidermal cells with surface secretion and subepidermal parenchyma (unstained, hand-cut section,). **(E)** Surface of epidermis with nectar residue (arrow). **(F)** Section stained with IKI; note starchless plastids. **(G)** Longitudinal section of cuniculus, the lumen visible at its top. Autofluorescence of chlorophyll located in subepidermal and ground parenchyma on exposure to UV. **(H)** Thin cuticle of epidermal cells with secretory residues. Beneath the epidermis occur large, thin walled parenchyma cells (auramine O). Scale bars: A = 2 mm; B = 500 μm; C = 200 μm; D,G = 100 μm; E,F,H = 50 μm.

in the cuniculus (**Figure 5D**). This region lay adjacent and parallel to the transmitting tract and ovary. The dorsal position of secretory tissue was observed only in *E. vesicatum*. The remaining area inside the cuniculus was non-secretory. Nectary tissue was composed of epidermal cells and subepidermal parenchyma. Epidermal cells enclosing the cuniculus in the nectary region were convex with large, centrally located vacuoles and parietal cytoplasm (**Figures 5E,F**). These cells had thin walls and a thin layer of smooth cuticle (**Figure 5E**), in contrast to the non-secretory area of the cuniculus, where cell walls were associated with a thicker layer of cuticle (not shown). Treatment with IKI revealed the absence of starch grains in nectary cells

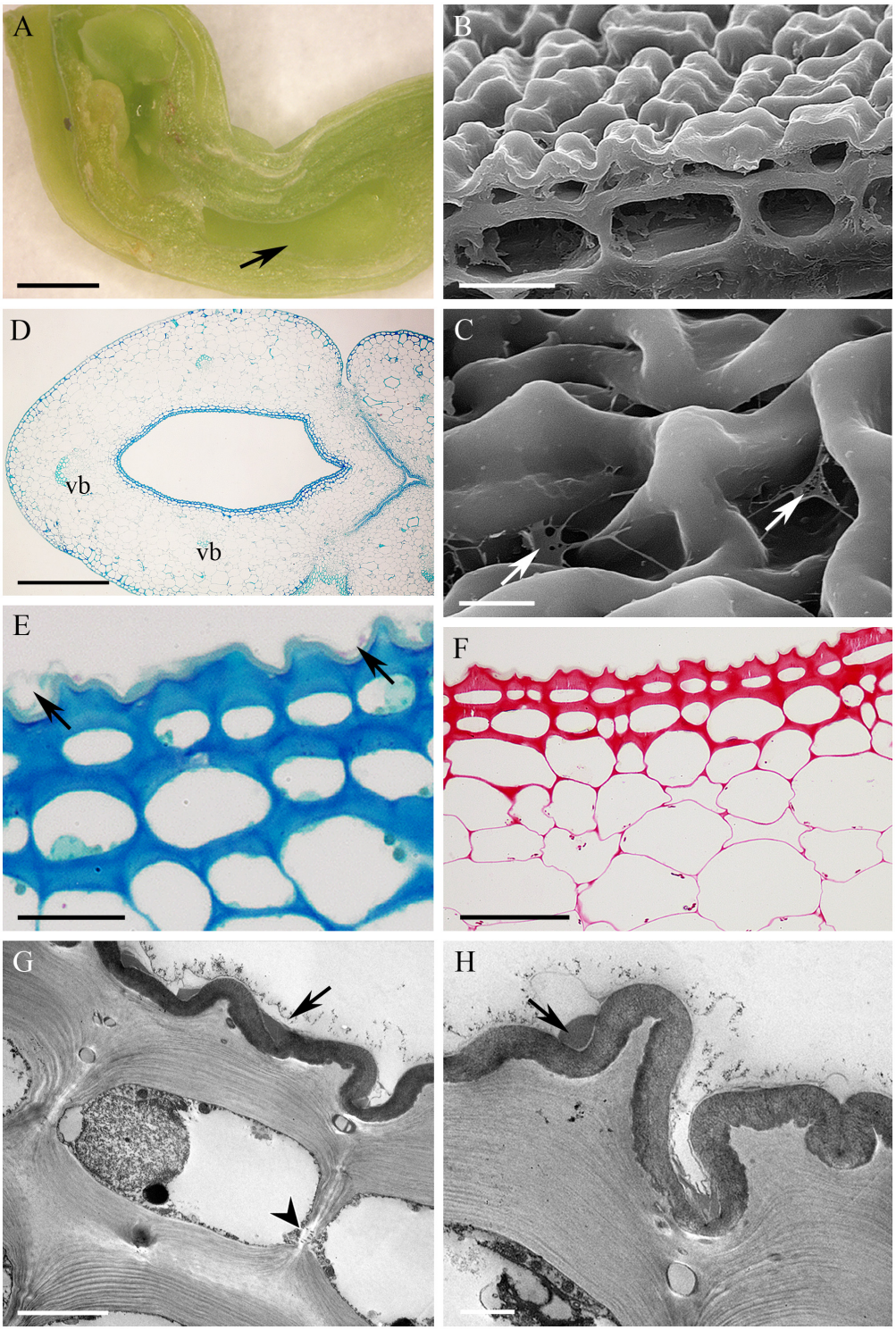

FIGURE 4 | *Epidendrum rigidum*. **(A)** Longitudinal section of flower showing cuniculus (arrow). **(B)** Detail of small, thick-walled epidermal cells with convoluted cuticle, and larger subepidermal parenchyma cells. **(C)** Detail of cuticle with nectar residues (arrows). **(D)** Transverse section of ovary showing cuniculus enclosed by epidermis and parenchyma containing vascular bundles (MB/AII). **(E)** Detail of collenchymatous epidermis and subepidermal parenchyma. Note thin cuticle with secretory residues (arrows). **(F)** The PAS reaction stains cell walls and occasional starch grains in ground parenchyma. **(G)** Epidermal cell lining cuniculus, with large nucleus and parietal cytoplasm containing osmiophilic globules. Secreted surface material occurs on the cuticle (arrow). Plasmodesmata in anticlinal cell wall marked with arrowhead. **(H)** Detail of outer wall of epidermal cell lining cuniculus, showing cuticle with nectar residues (arrow) and cavity in middle lamella. Scale bars: A = 1mm; B,C,E = 20 μm; D,G = 5 μm; D = 500 μm; F = 50 μm; H = 2 μm.

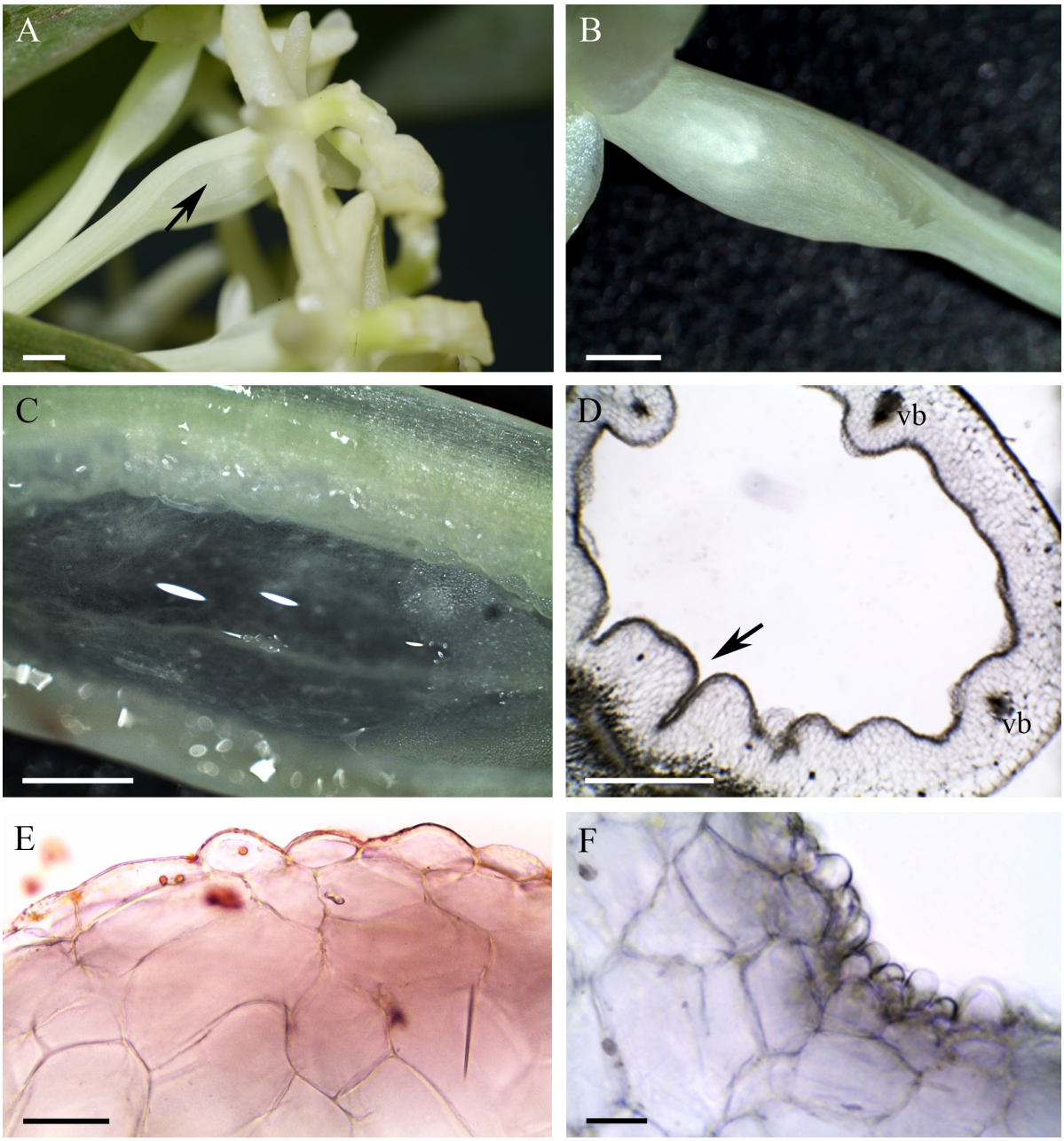

FIGURE 5 | *Epidendrum vesicatum*. **(A)** Habit of the flower, cuniculus marked by arrow. **(B)** Lateral view of cuniculus. **(C)** Longitudinal section of cuniculus containing copious nectar. **(D)** Transverse section of cuniculus stained with IKI showing secretory tissue (arrow) adjacent to the transmitting tract. **(E)** Transverse section of secretory tissue stained with Sudan III. Note the thin cuticle present on secretory cells. **(F)** Detail of transverse section stained with IKI; note papillose epidermal cells and the absence of starch grains. Scale bars: A,B = 2mm, C,D = 1 mm; E,F = 20 μm.

(**Figure 5F**). Three collateral vascular bundles supplied the ground parenchyma of the cuniculus (**Figure 5D**).

Nectarless Species With no Nectar Visible on Macroscopic Observation

The cuniculus of the non-fragrant, pink flowers of *Epidendrum capricornu* (**Figure 6A**) was wide at its entrance and tapered toward its base (**Figure 6B**), and measured 15 mm in length. The epidermal cells were conical close to the entrance, but papillose toward the base of the cuniculus, particularly on the side adjacent to the transmitting tract (**Figures 6C–G,I**). The striate cuticle of epidermal cells (0.90 μm ± 0.15 thick) lacked pores, but copious globular blisters were visible on its surface, when viewed by SEM and TEM (**Figures 6C,H**). Blisters with underlying material were also visible in sections

stained with auramine O (**Figure 6I**). The tangential walls of both epidermal cells and the underlying parenchyma cells were cellulosic and thick (2.54 μm ± 1.02), but toward the tapered end of the cuniculus, cell walls were thinner. TEM observations indicated the presence of intravacuolar electron-dense, phenolic-like material (not shown). Similarly, electron-dense material was also observed to occur between the cellulosic microfibrils of the outer, tangential cell wall, and beneath the blistered cuticle (**Figure 6H**). Both epidermal and subepidermal parenchyma cells possessed a large central vacuole and a thin layer of parietal cytoplasm (**Figures 6E,I**), and accumulated starch (**Figure 6F**). The cuniculus was supplied with three collateral vascular bundles (**Figure 6D**).

The cuniculus of the white, fragrant flowers of *Epidendrum ciliare*, measured 45 mm in length (**Figure 7A**). Epidermal cells enclosing the cuniculus were flattened at its entrance and papillose toward its base (**Figures 7B–F**). The cuticle present on the epidermal papillae was ridged at their apices, but finely striate on the sides of the papillae (**Figures 7C,D**), and was 1.99 μm ± 0.52 thick. Despite the apparent absence of nectar during macroscopic investigations, surface secretion that resembled nectar and that coated the apical parts of the papillae was visible under SEM (**Figure 7D**). It was also observed by TEM to collect beneath the cuticular distensions (**Figures 7G,H**). The epidermal cells and the underlying 3-4 layers of parenchyma cells were smaller than those of the ground parenchyma cells through which ran several vascular bundles. In transverse section, epidermal cells and several layers of subepidermal cells were seen to possess thick (4.44 μm ± 0.99) tangential, cellulosic walls (**Figures 7E,F,H,I**), Such walls were particularly pronounced opposite the transmitting tract (**Figure 7B**). Numerous primary pit-fields with plasmodesmata in anticlinal and periclinal walls connected epidermal and subepidermal parenchyma cells (**Figures 7E,I**). TEM investigations showed the cuticle to be bilayered, having an outer lamellate layer and inner electron-dense and reticulate layer. Both these layers were highly convoluted (**Figure 7H**). The protoplasts of epidermal and subepidermal cells were electron-dense and contained numerous mitochondria, dictyosomes, ER profiles and secretory vesicles (**Figures 7I,J**). Small vacuoles containing vesicles or flocculent material were present (**Figure 7J**), and the larger vacuoles of the ground parenchyma had similar contents. The plastids contained an electron-dense stroma and few lamellae. Generally, these last organelles did not contain starch, but occasionally, starch grains were observed in parenchyma cells adjacent to vascular bundles. Chloroplasts were abundant in ground parenchyma cells. Numerous collateral vascular bundles of variable size were scattered throughout the ground parenchyma (**Figure 7B**). Lipids were detected exclusively in the cuticular layer (**Figure 7F**).

Flowers of *Epidendrum criniferum* lacked fragrance, were greenish-white and spotted with magenta. The cuniculus measured 15.2 mm in length. It formed a wide reservoir below the entrance (**Figure 8A**) and tapered distally. The cuniculus was lined with flat or slightly convex epidermal cells that possessed a convoluted or ridged cuticle (**Figures 8B–H**) 1.24 μm ± 0.23 thick. Traces of secretory material were visible on the surface of the cuticle, when viewed by SEM (**Figure 8B**). The epidermal cells had thick tangential walls (4.34 μm ± 1.03), whereas those of the subepidermal and ground parenchyma were thin (**Figures 8D–G**). Epidermal and subepidermal cells contained a narrow layer of parietal cytoplasm and a large, central vacuole containing globular material (**Figure 8G**). Strands of cellulosic wall microfibrils occurred beneath the cuticular ridges (**Figure 8H**). Starch was present in both subepidermal and deeply located ground parenchyma cells (**Figure 8E**), whereas chloroplasts occurred only in the latter. The cuniculus was supplied with three collateral vascular bundles (**Figure 8C**).

The cuniculus of the orange and green, non-fragrant flowers of *Epidendrum pseudepidendrum* was 38 mm long. It had a very narrow entrance expanding to form a wider region at the level of insertion of the perianth segments (**Figure 9A**). The epidermis at the mouth of the cuniculus was papillose, the papillae being longer toward its base (**Figures 9B–G**). The cuticle overlying the papillae was thick (0.45 μm ± 0.06) and formed blisters and distensions (**Figures 9C,E**). Surface secretory material was present apically and between cuticular ridges (**Figures 9E,F,H,I**). This material, which stained with Sudan III, was also present in intercellular spaces (**Figure 9G**). The walls of epidermal cells and 1-2 layers of the subepidermal tissue were 1.57 μm ± 0.30 thick and cellulosic (**Figures 9D–I**). Three large and several small collateral vascular bundles supplied the ground parenchyma (**Figure 9D**). Observations using TEM revealed that epidermal cells contained a large nucleus and electron-dense, granular cytoplasm with mitochondria and secretory vesicles, the last fusing with the plasmalemma (**Figure 9I**). Plastids with starch and/or an electron-dense stroma were present in subepidermal and ground parenchyma cells (**Figures 9F,J**), whereas chloroplasts occurred only in ground parenchyma cells. Lipid bodies were occasionally observed in epidermal cells. Primary pit-fields with plasmodesmata (**Figure 9J**) were present in periclinal walls between epidermal and subepidermal cells.

The cuniculus of the non-fragrant, orange flowers of *Epidendrum radicans* measured 25 mm in length. The epidermal cells at its entrance were papillose. Of the investigated taxa, this species was unique in that the cuniculus was lined with unicellular trichomes of average length 132 μm. These epidermal trichomes arose from just below the entrance to the cuniculus and were distributed along its length to the base (**Figures 10A–I**). They had a smooth and thick (1.55 μm ± 0.89) cuticle (**Figures 10C,H**). Observations of the cuticle by means of SEM, LM and TEM revealed the presence of surface material, as well as cuticular distensions (**Figures 10C,K**). Cell walls of trichomes were 1.21 μm ± 0.25 thick. Epidermal and subepidermal cells were small compared with those of the underlying ground parenchyma, the cell walls being only slightly thickened (**Figures 10D–F,I**). These walls were 0.91 μm ± 0.19 thick and had a thin cuticle (0.26 μm ± 0.07). The epidermal cells, including the unicellular trichomes, had dense protoplasts containing a large nucleus and small vacuoles (**Figures 10E,H–J**). Mitochondria, ER profiles, dictyosomes and secretory vesicles were predominant in the cytoplasm of trichomes and subepidermal parenchyma cells. The plastids contained an electron-dense stroma, densely packed stacks of lamellae and plastoglobuli, but no starch. Starch, however, was

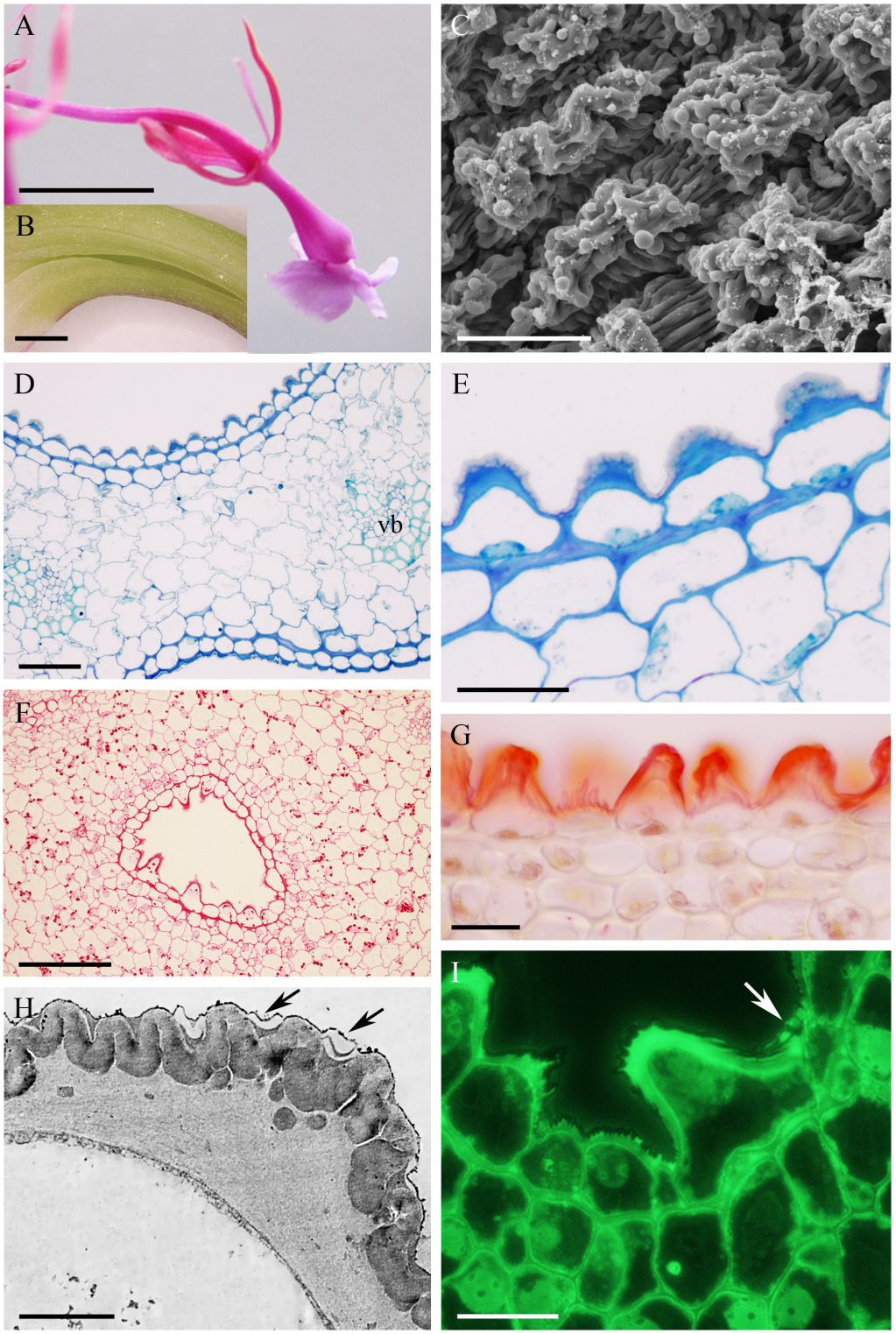

FIGURE 6 | *Epidendrum capricornu*. **(A)** Habit of the flower. **(B)** Longitudinal section showing cuniculus. **(C)** Globular blisters on epidermal cells lining cuniculus. **(D)** Conical cells of epidermis enclosing cuniculus and parenchyma with vascular bundles (MB/All). **(E)** Detail showing epidermis and subepidermal parenchyma. Note the thick, tangential walls of the epidermal cells and the convoluted cuticle. **(F)** PAS reaction stains copious starch present in papillose epidermis and subepidermal parenchyma. **(G)** Cuticle stained with Sudan III. **(H)** Outer epidermal cell wall with globular electron-dense material between cellulosic microfibrils, and blistered cuticle (arrows). **(I)** Section showing conical, epidermal cells and subepidermal parenchyma. Globules of secretion indicated by arrow. Scale bars: A = 1 cm; B = 1 mm; C,E,G,I = 20 μm; D = 50 μm; F = 200 μm; H = 2 μm.

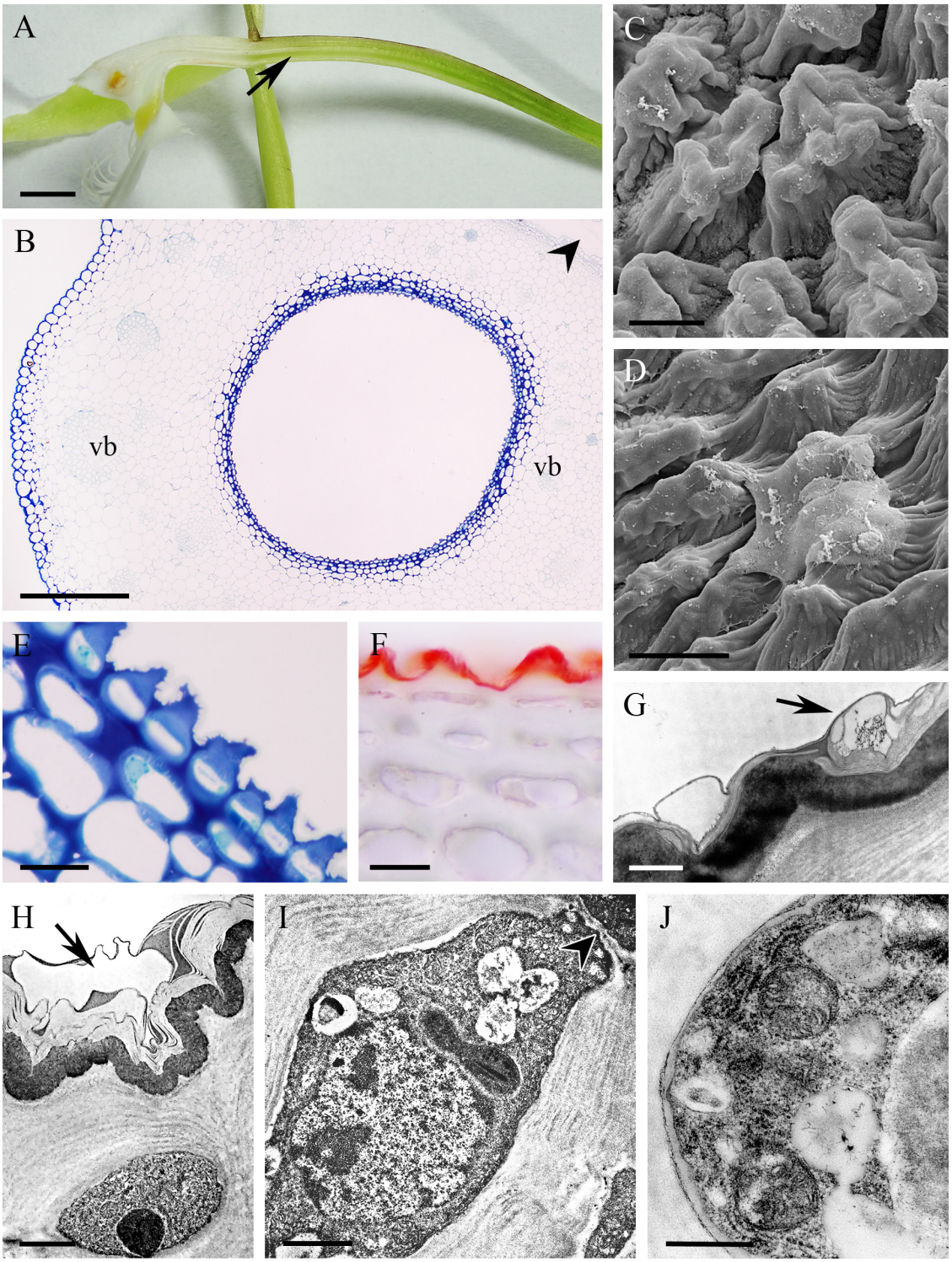

FIGURE 7 | *Epidendrum ciliare.* **(A)** Longitudinal section of flower. Cuniculus indicated by arrow. **(B)** Transverse section of ovary with cuniculus and surrounding parenchyma containing vascular bundles (MB/AII). Transmitting tract is indicated by arrowhead. **(C)** Papillose epidermal cells lining cuniculus. **(D)** Secretion coating epidermis. **(E,F)** Epidermis and subepidermal parenchyma of cuniculus (MB/AII and Sudan III, respectively). Note thick tangential cell walls and cellulose projections penetrating cuticle. **(G)** Cell wall with thick, bilayered cuticle and cuticular blisters containing secretion (arrow). **(H)** Surface secretion and cuticular blister (arrow) of epidermal cell. **(I)** Electron-dense protoplast of epidermal cell containing large nucleus, starchless plastid and small vacuoles with flocculent content. Plasmodesmata in anticlinal cell wall marked with arrowhead. **(J)** Detail of parietal cytoplasm with profiles of ER, mitochondria and secretory vesicles. Scale bars: A = 1 cm; B = 500 μm; C = 10 μm; D–F = 20 μm; G = 1 μm; H, I = 2 μm; J = 0.5 μm.

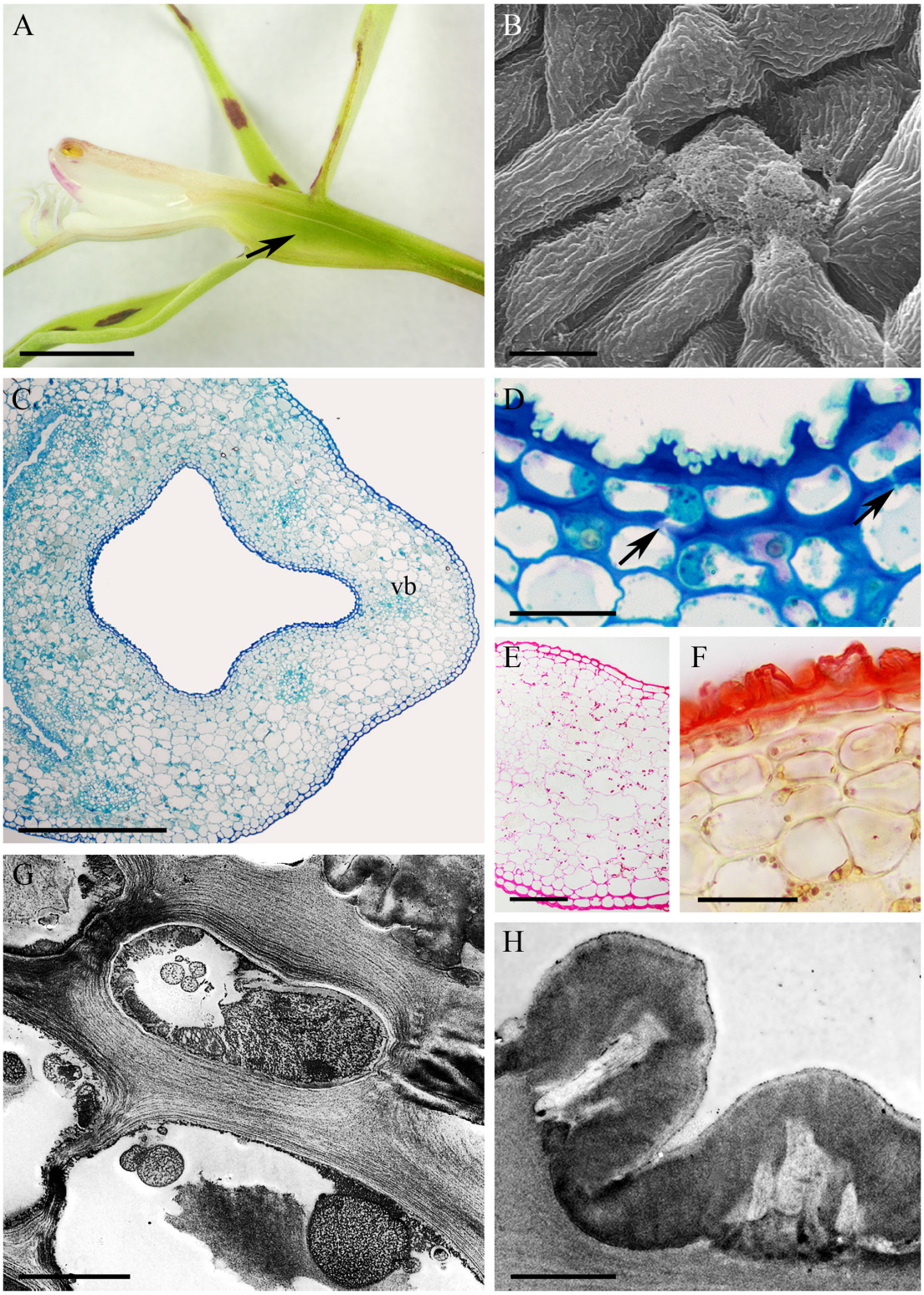

FIGURE 8 | *Epidendrum criniferum*. **(A)** Longitudinal section of flower, the expanded part of the cuniculus indicated by arrow. **(B)** Surface secretion on cuticle. **(C)** Transverse section of ovary showing cuniculus and surrounding tissues (MB/AII). **(D)** Epidermis and subepidermal parenchyma with primary pit-fields (arrows). Cellulosic projections of cell wall with overlying cuticle (MB/AII). **(E)** PAS reaction shows starch in subepidermal parenchyma. **(F)** Thick cuticle stained with Sudan III. **(G)** Epidermal and subepidermal cells with intravacuolar, globular material. **(H)** Convoluted cuticle with cellulosic projections of cell wall. Scale bars: A = 4 mm; B = 50 μm; C = 500 μm; D = 20 μm; E = 100 μm; F = 30 μm; G = 5 μm; H = 2 μm.

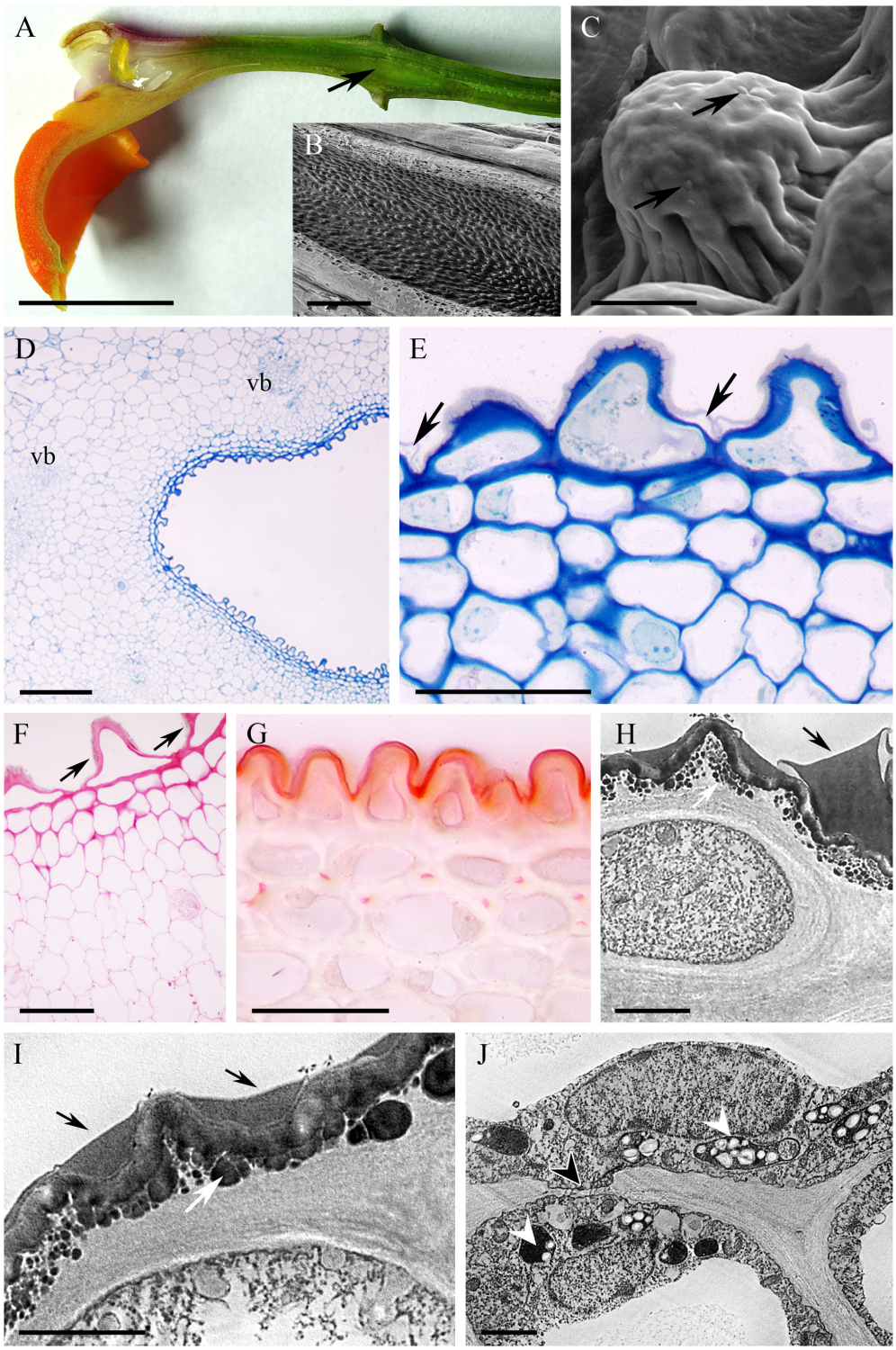

FIGURE 9 | *Epidendrum pseudepidendrum*. **(A)** Longitudinal section of flower, cuniculus indicated by arrow. **(B)** Papillose epidermal cells lining cuniculus. **(C)** Detail of epidermal cell with cuticular blisters (arrows). **(D)** Epidermis and parenchyma with vascular bundles enclosing cuniculus (MB/AII). **(E)** Detail of epidermis and subepidermal parenchyma of cuniculus. Note secreted material beneath cuticle (arrows). **(F)** PAS reaction stains cell walls and starch in ground parenchyma cells. Surface material accumulates between cuticular ridges (arrows). **(G)** Sudan III stains cuticle and intercellular material. **(H)** Epidermal cell with surface secretion (arrow). **(I)** Detail of epidermal cell wall with subcuticular globular material (white arrow) and surface secretion (black arrows). Secretory vesicles fusing with plasmalemma are also visible. **(J)** Parenchyma cell with plastids containing an electron-dense stroma and starch grains (arrowheads). A primary pit-field with plasmodesmata marked with arrowhead. Scale bars: A = 1 cm; B,D = 200 μm; C = 10 μm; E,G = 40 μm; F = 50 μm; H,J = 2 μm.

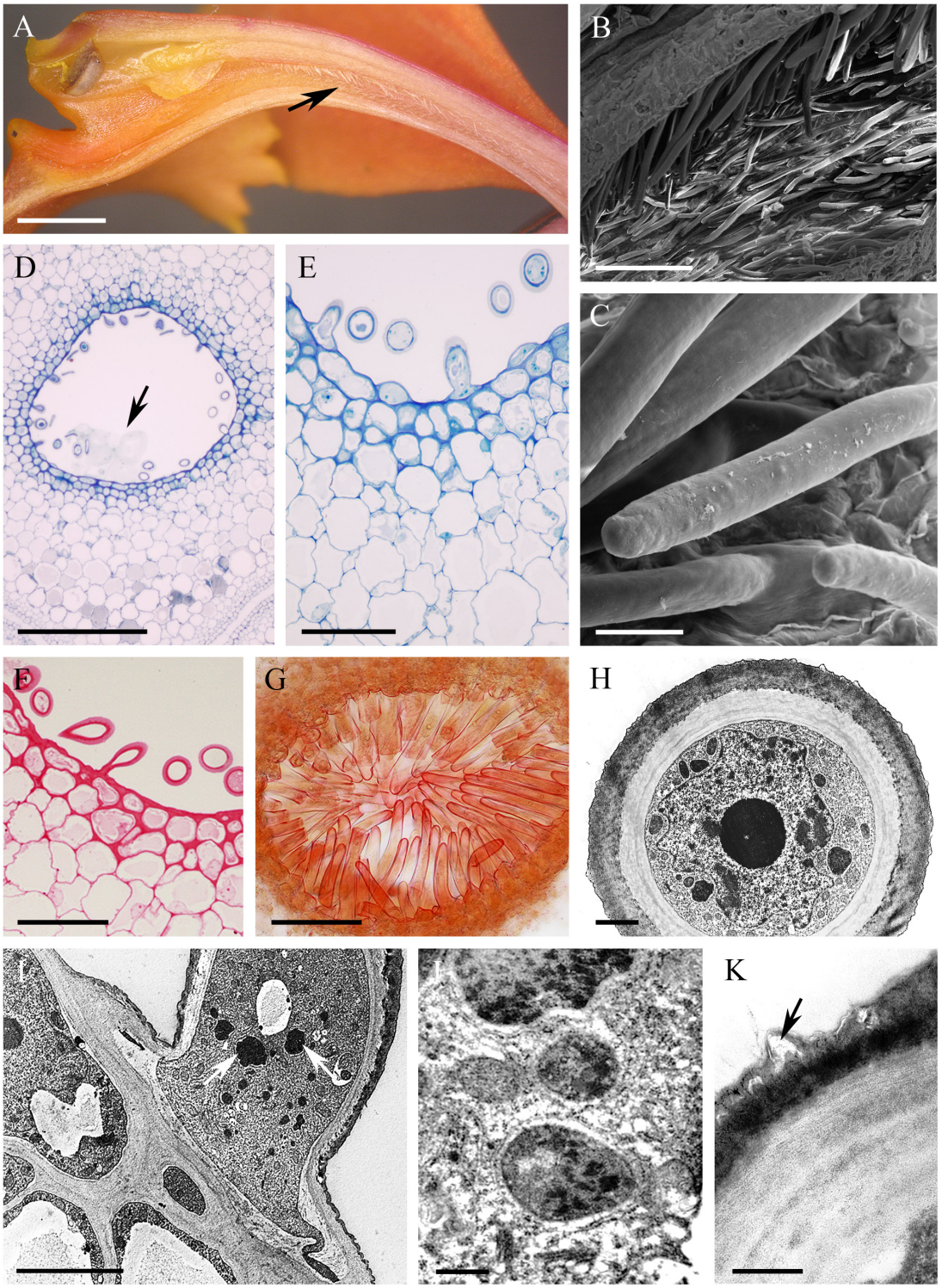

FIGURE 10 | *Epidendrum radicans*. **(A)** Longitudinal section of flower, the cuniculus indicated by an arrow. **(B)** Unicellular trichomes lining cuniculus. **(C)** Detail of smooth cuticle of trichomes with small blisters. **(D)** Tissues enclosing cuniculus. Secreted surface material is marked by arrow (MB/AII). **(E)** Detail of epidermis of cuniculus with trichomes and subepidermal tissues. **(F)** PAS reaction stains cellulosic cell walls. **(G)** Sudan III stains thick cuticle of trichomes. **(H)** Transverse section of trichome showing cell wall with thick, smooth, but slightly blistered cuticle and electron-dense protoplast enclosing large nucleus. **(I)** Longitudinal section through trichome, epidermal and subepidermal cells. Note electron-dense plastids (arrows) in trichome. **(J)** Detail of cytoplasm of trichome with starchless plastids, mitochondria, dictyosomes and ER. **(K)** Cell wall of trichome with blistered cuticle (arrow). Scale bars: A = 2 mm; B = 200 μm; C = 20 μm; D = 500 μm; E,F = 50 μm; G = 100 μm; H = 2 μm; I = 5 μm; J,K = 0.5 μm.

present in the plastids of ground parenchyma. Chlorophyll was not detected by FM in parenchyma cells surrounding the cuniculus. The ground parenchyma was supplied by three collateral vascular bundles (not shown).

The cuniculus of the non-fragrant, yellow, rose and green flowers of *Epidendrum xanthoianthinum* measured 14 mm long. Its entrance was wide, but the lumen tapered toward the base (**Figure 11A**). The epidermis enclosing the cuniculus was composed of slightly convex cells possessing a convoluted or ridged cuticle (**Figures 11B–G,I**) 1.87 μm ± 0.27 thick. Abundant cuticular blisters were visible under SEM (**Figure 11B**), and in TEM, these appeared electron-translucent (**Figures 11G,I**). The tangential cellulosic walls of the epidermal cells and one layer of the subepidermal parenchyma were slightly thickened (**Figures 11D–H**), those of the epidermis being 2.28 μm (±0.62) thick. The epidermal cells contained intensely staining cytoplasm, together with a large nucleus and plastids containing small starch grains (**Figures 11D,E,G**). Moreover, TEM investigations revealed the presence of numerous lamellae and plastoglobuli (**Figure 11H**) within these plastids. The cytoplasm also contained arrays of ER, as well as dictyosomes and secretory vesicles (**Figures 11G,H**). The tissues surrounding the cuniculus were supplied with three large, and several small collateral vascular bundles (**Figure 11C**).

DISCUSSION

Species of *Epidendrum* investigated in this study varied greatly in terms of flower size, color, fragrance, and also in the size and shape of the cuniculus. All these characters may reflect the type of pollinator associated with each species. Whereas some of the flowers are obviously nectariferous and produce copious nectar (*E. difforme*, *E. nocturnum* and *E. vesicatum*), others produce smaller volumes of nectar (*E. porpax* and *E. rigidum*), whereas yet others seemingly produce none (*E. capricornu*, *E. ciliare*, *E. criniferum*, *E. pseudepidendrum*, *E. radicans*, *E. xanthoianthinum*). Although evidence for nectar secretion has previously been recorded for Laeliinae, the amount produced is frequently small, making it difficult to measure volume and sugar concentration (Krahl et al., 2017). Conversely, *E. vesicatum* and *E. nocturnum* produce copious amounts of nectar, which can be easily collected with micro-syringes, and its sugar concentration measured with a refractometer. *Epidendrum vesicatum*, whose flowers are adapted to pollination by nocturnal moths, produces 9–15 μL of dilute nectar of concentration 5–7% sugar (E.R. Pansarin, unpublished data).

In the absence of a nectar reward, approximately one-third of investigated orchid species rely on various kinds of deception or mimicry to attract pollinators (Cozzolino and Widmer, 2005; Jersáková and Johnson, 2006; Gaskett, 2011), and many reports have indicated that deception was the ancestral condition in Orchidaceae (Hobbhahn et al., 2013; Johnson and Schiestl, 2017, and references therein). According to Cozzolino and Widmer (2005) and Jersáková et al. (2006), deception can, under certain circumstances, be advantageous in that it enables conservation of resources and discourages repeated visits by pollinators, thereby promoting outcrossing. Food-deception has been reported for Laeliinae, including *Epidendrum* (Boyden, 1980; Almeida and Figueiredo, 2003; Pansarin and Amaral, 2008b; Fuhro et al., 2010; Vega and Marques, 2015). Indeed, according to Ackerman (1986), *Epidendrum* is primarily a food deceptive genus. This is supported by the work of Hágsater and Soto-Arenas (2005) which reports that many members of *Epidendrum*, e.g., the *Epidendrum secundum* complex, do not offer any nectar reward, with some observations indicating that many *Epidendrum* spp. display attributes of food-deceptive taxa, such as infrequent visits by pollinators and low fruit set (Ackerman and Montalvo, 1990; Almeida and Figueiredo, 2003; Pansarin and Amaral, 2008b; Fuhro et al., 2010; Pinheiro et al., 2010, 2011). Therefore, in future, it will be necessary to investigate nectar production by certain taxa, including members of the *Epidendrum secundum* complex, more thoroughly and critically, since it is now known that the flowers of some species of *Epidendrum*, once thought to employ nectar deception pollination strategies (e.g., *E. puniceoluteum*), in fact, have nectar-secreting epidermal papillae, and that nectar is collected from them by both hummingbirds and butterflies (E.R. Pansarin, pers. obs.). Remarkably, some species of *Epidendrum* that lack floral nectar possess extra-floral nectaries. The production of extra-floral nectar is generally considered a defense strategy in that it encourages ants to patrol plants, thus discouraging herbivory (Delabie, 1995; Almeida and Figueiredo, 2003). Even so, since *E. nocturnum* produces copious nectar, yet was the only species investigated in this study to have extra-floral nectaries, their presence is clearly not restricted exclusively to non-rewarding species.

The rewardless status and deceptive pollination systems proposed for a number of species on the basis of macroscopic observations alone is entirely understandable since, in fact, no nectar whatsoever was visible using this technique for *E. capricornu*, *E. criniferum*, *E. pseudepidendrum*, *E. radicans*, and *E. xanthoianthinum*. Nevertheless, close observations of cells lining the cuniculus in presumed rewardless species by LM, SEM and TEM revealed the presence of nectar-like residues and established that they possess an organelle complement typical of secretory cells. As well as similarities in the ultrastructure of these cells and the presence of thick tangential cell walls (with the exception of *E. vesicatum*), other secretory characters particularly pronounced in *E. nocturnum, E. ciliare, E. radicans* and *E. xanthoianthinum* included the abundant mitochondria, ER and secretory vesicles. Furthermore, cell wall protuberances were present in *E. nocturnum,* indicating intense transmembrane transport. The cuticle overlying the epidermal cells lining the cuniculus in these species was blistered, and secretory material had accumulated beneath and upon its surface. No relationship was found to occur between the thickness and structure of the cuticle in both species with copious nectar occurring in the cuniculus, and those exhibiting only residues of secreted surface material. Of the investigated species, only the cuniculus of *E. radicans* was lined with unicellular trichomes, and in this respect, it resembled that of *E. fulgens*, whose anatomy was studied by Moreira et al. (2008). These authors proposed, owing to the dense cytoplasmic content of the trichomes, that

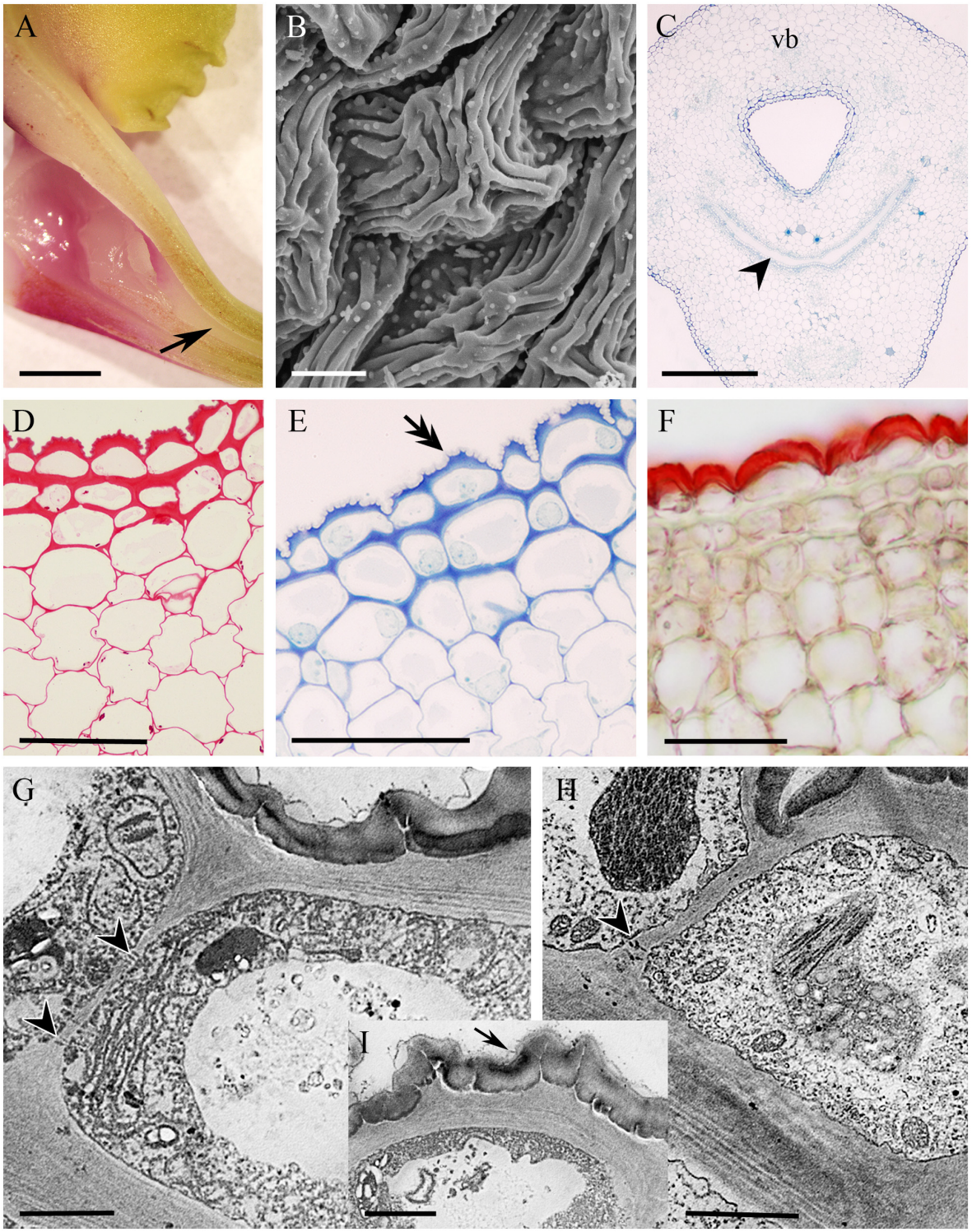

FIGURE 11 | *Epidendrum xanthoianthinum*. **(A)** Longitudinal section of flower, the cuniculus indicated by arrow. **(B)** Ridged surface of blistered cuticle of epidermal cells. **(C)** Transverse section of ovary showing cuniculus and associated tissues, the transmitting tract indicated by arrowhead (MB/AII). **(D)** PAS reaction stains cell walls and sparse starch grains. **(E)** Detail of epidermis and subepidermal parenchyma. Note that cellulosic projections of the cell wall (double arrow) penetrate the cuticle (MB/AII). **(F)** The thick cuticle stains selectively with Sudan III. **(G)** Parietal cytoplasm of epidermal cell, showing profiles of ER and plastids. Vesicles are present in the vacuole. Surface secretion is visible on the cuticle. **(H)** Plastids with dense stroma and minute starch grains in epidermal cells. In **G,H**, plasmodesmata in anticlinal walls are marked with arrowheads. **(I)** Detail of cell wall penetrating cuticle and secreted surface material (arrow). Scale bars: A = 2 mm; B = 10 μm; C = 300 μm; D–F = 50 μm; G–I = 2 μm. vb, vascular bundle.

the latter are highly metabolically active and thus, probably involved in secretion, even though no nectar was found within the cuniculus. Similarly, in *E. radicans*, the organelle complement of such trichomes, coupled with the presence of surface material, indicated that they too are secretory.

We did not find any floral food-rewards other than nectar in species investigated in this study. In our opinion, the presence of nectar-like surface residues during detailed microscopical investigations is indicative of, at the very least, a limited degree of nectary activity, and it may be that meager volumes of nectar are sufficient to maintain the interest of pollinators. According to Ackerman and Montalvo (1990), *E. ciliare* is self-compatible, but outcrossed and pollinated by the moth *Pseudosfinx tetrio*. During experimentally augmented pollination, fruit-set increased in the short-term, but in subsequent seasons, it declined significantly, since greater fruit production demanded greater resources. Consequently, a large number of pollination events and investment in the production of large volumes of nectar do not always benefit the plant. Another explanation for the absence of nectar from the cuniculus of species predominantly visited by nocturnal pollinators is that nectar secretion occurs only at night and is reabsorbed during the day. In fact, based on floral characters and the release of fragrance at night, many species of *Epidendrum* are believed to be pollinated by nocturnal moths (van der Pijl and Dodson, 1969; Pansarin and Pansarin, 2010), and this has been confirmed by investigations of their reproductive biology (Pansarin, 2003; Pansarin and Pansarin, 2017).

Relatively numerous globular blisters were observed by SEM on the cuticle of epidermal cells lining the cuniculus of *E. capricornu* and *E. xanthoianthinum*. TEM observations indicated that they are delimited delimited by a thin layer of cuticle bearing almost electron-translucent material that probably represents nectar. It is thus likely that the abundant cuticular blisters present in these two species were probably the result of epidermal secretory activity.

Epidendrum pseudepidendrum is regarded to be a hummingbird-pollinated and rewardless species (van der Pijl and Dodson, 1969; van der Cingel, 2001). Nevertheless, as far as we are aware, there are no experimental data to support this assertion. If, however, this is true, it would pose an interesting conundrum, since bird-pollinated flowers usually offer nectar. Although we demonstrated the presence of surface secretion in this species, owing to its osmiophilic nature, this secretion evidently is not a simple sugar solution. Nectar, far from being merely a dilute aqueous solution of sugars, may also contain other compounds such as amino acids, lipids and secondary metabolites, some of which are osmiophilic. Therefore, we cannot rule out the possibility that *E. pseudepidendrum* is indeed nectariferous.

In most of the obviously nectariferous taxa investigated, the epidermis of the cuniculus was relatively glabrous, whereas in seemingly nectarless species, it was predominantly papillose or trichomatous. This is not congruent with the nectary studies undertaken for some members of Orchidoideae, where nectar secretion was shown to be positively correlated with the presence of papillae (Bell et al., 2009). Trichomes and papillae were also present in the nectaries of other genera of Epidendroideae, such as *Oeceoclades* (Aguiar et al., 2012), *Ascocentrum* (Stpiczyńska et al., 2011), and representatives of Laeliinae, such as *Encyclia* (Krahl et al., 2017) and *Brassavola flagellaris* (Stpiczyńska et al., 2010). The presence of epidermal papillae and trichomes has been considered a strategy for increasing the surface area for nectar secretion/reabsorption (Stpiczyńska, 2003; Stpiczyńska et al., 2005). Since it is likely that all species of *Epidendrum* investigated in this study secrete nectar to a greater or lesser degree, it is not possible to correlate nectar production with the presence of papillae/trichomes. Nevertheless, the possibility that the increased surface area of the epidermis lining the cuniculus may account for the seemingly nectarless status of certain species, cannot be discounted, since the secreted nectar may be reabsorbed more efficiently. It is worth stating, however, that the presence of papillae is not necessarily exclusively related to nectar secretion/reabsorption. For example, papillae present in the spur of deceptive orchids such as *Dactylorhiza* (Bell et al., 2009) probably provide tactile cues for insect visitors.

In all investigated species (with the exception of *E. vesicatum*), epidermal and subepidermal parenchyma cells had thick, tangential cellulosic cell walls, and in the case of the outer epidermal walls, cellulosic projections extended as far as, and traversed the thick cuticle, possibly facilitating the transport of secretion across the latter. Such thick cellulosic walls are characteristic of collenchyma. Collenchymatous cell walls have also been recorded for the nectaries of other species of *Epidendrum* (e.g., Pansarin and Amaral, 2008b; Vieira et al., 2017), the cuniculus of *Brassavola flagellaris* (Stpiczyńska et al., 2010), the nectaries of putatively ornithophilous *Maxillaria coccinea* (Stpiczyńska et al., 2004) and *Ornithidium sophronitis* (Stpiczyńska et al., 2009), and some Aeridiinae (Stpiczyńska et al., 2011). Here, collenchyma may prevent mechanical damage to the nectary tissues, and also facilitate apoplastic transport of nectar. Numerous pit-fields with plasmodesmata traversing anticlinal cell walls between epidermal cells, as well as periclinal walls between epidermal and parenchyma cells, may also be indicative of symplastic transport. Abundant plasmodesmata connections have also been reported for the nectary tissues of other plant species having thick collenchymatous cell walls, such as *M. coccinea* (Stpiczyńska et al., 2004), as well as those with thin-walled nectary cells (e.g., Nepi, 2007; Stpiczyńska et al., 2011, 2018). Our results generally agree with the model proposed by Vassilyev (2010) for the functioning of the nectary. According to this author, nectar moves by a pressure-driven mass flow along an apoplastic route, but pre-nectar sugars are transported from the phloem via the symplast to the secretory cells, where finally, nectar is formed, and sugars are actively transported across the plasmalemma by eccrine secretion. Since abundant secretory vesicles were present in secretory cells of the investigated species, both close to the plasmalemma and in the parietal cytoplasm, we propose that granulocrine secretion also operates in nectary cells of *Epidendrum*.

The thick cell walls of *E. nocturnum* were particularly remarkable in their possession of wall protuberances thought to improve efficient transport across the plasmalemma. Although cell wall protuberances have frequently been reported for the nectaries of other angiosperms (Fahn, 1979;

Kronestedt-Robards and Robards, 1991), they have rarely been recorded for Orchidaceae (Pais and Figueiredo, 1994; Stpiczyńska et al., 2018). The involvement in intense secretory activity of the epidermal cells enclosing the cuniculus of investigated species of *Epidendrum* is confirmed by the presence of numerous secretory vesicles that gather next to the plasmalemma, the extensive arrays of endoplasmic reticulum, the abundant mitochondria, and the dictyosomes, as well as invaginations of the plasmalemma that increase the surface area for secretion and possible reabsorption of nectar. In future, we intend performing ultrastructural studies on the thin-walled nectary cells of *E. vesicatum*, in order to assess how well they are structurally adapted for nectar secretion.

We did not measure the volume of secreted nectar in the present project, but based on microscopical observations, we found no connection between the number and distribution pattern of vascular bundles present in the parenchyma and secretory activity. However, the secretory status and nectary activity of all species investigated are further supported by the distribution of abundant starch predominantly located near the main vascular bundles supplying the nectary, but also, in some cases, within epidermal and subepidermal parenchyma cells. The importance of starch has been widely reported for the floral secretory tissues (in particular, nectaries) of many taxa, including orchids, and it has been proposed that hydrolysis of starch reserves provides both the metabolic energy for the secretory process and the sugars for nectar production (Pacini and Nepi, 2007). In the majority of investigated species, parenchyma cells containing chloroplasts were also able to synthesize sugars, whereas plastids within the secretory epidermis frequently possessed an electron-dense stroma, indicating that they might be engaged in the synthesis of various secondary metabolites, including alkaloids (Facchini, 2001), that are frequently present in nectar. Many species of *Epidendrum* are visited by butterflies. Some male lepidopterans are attracted by pyrrolizidine alkaloids that are used in mating (van der Cingel, 2001 and references therein; Hágsater and Soto-Arenas, 2005 and references therein) and in the synthesis of their pheromones. In many plant species, alkaloids are a common constituent of nectar (Masters, 1991; Adler and Irwin, 2005; Nepi, 2007; Manson et al., 2013), and since *E. difforme* is visited by male Ctenuchidae and Noctuidae moths (Goss, 1977), it is possible that its nectar also contains alkaloids. The occurrence of pyrrolizidine alkaloids has been considered a factor involved in attracting Ithomiinae butterflies to the flowers of *E. paniculatum* (van der Pijl and Dodson, 1969). According to Pansarin (2003), a species closely related to *E. paniculatum* (namely, *E. densiflorum*), is also pollinated by Ithomiinae butterflies and Arctiidae moths, and both types of insect have been reported to collect alkaloids from flowers. However, tests for alkaloids showed these compounds to be absent from flowers of *E. densiflorum* (Pansarin, 2003). Detailed chemical analysis of *Epidendrum* nectar is now necessary.

CONCLUSION

To conclude, we agree with Pinheiro and Cozzolino (2013) that the genus *Epidendrum* is an ideal model system for the comparative study of the association between pollination efficiency and the evolution of floral traits in both rewarding and deceptive orchids, especially since members of this enormous genus display diverse reproductive systems ranging from self-incompatibility to autogamy (Hágsater and Soto-Arenas, 2005). For example, autogamy has been reported for nectariferous *E. nocturnum* (Catling, 1990) and for *E. rigidum* (Iannotti et al., 1987). By contrast, self-incompatibility occurs in nectariferous *E. difforme* (Goss, 1977), whereas in self-compatible taxa, geitonogamy is thought to be restricted to species that do not offer any food-rewards to pollinators (Almeida and Figueiredo, 2003; Pansarin and Amaral, 2008b; Fuhro et al., 2010). Nevertheless, as our study indicates, perhaps the time has come to look more closely at whether *Epidendrum* spp. considered to lack food-rewards merely on the basis of macroscopic examination really are rewardless, and to investigate their floral biology in association with molecular studies, in order to explore the evolution and the production of floral food-rewards in this genus (Pansarin et al., 2012; Pansarin and Maciel, 2017). Furthermore, this should not be restricted to *Epidendrum*, but extended to other orchid genera also.

AUTHOR CONTRIBUTIONS

MS conceived the study, did the microscopy, prepared the draft version of manuscript, and contributed to the final version. MK contributed to the microscopy and documentation, and the final figures. KD developed, expanded, and contributed to the final version of manuscript. EP contributed to the microscopy and contributed to the final version of manuscript.

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Functional Diversity of Nectary Structure and Nectar Composition in the Genus *Fritillaria* (Liliaceae)

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Fritillaria is a genus consisting of 130 to 140 species of bulbous plants, native to temperate regions of the northern hemisphere. Generally viewed as an insect pollinated genus with the exception of two North American species, *Fritillaria gentneri* and *F. recurva*, which are described as hummingbird-pollinated and the Asian species, *F. imperialis*, described as passerine-pollinated. These pollinator shifts are possibly the result of adaptive changes to the structure and morphology of the nectary, as well as a change in the nectar concentration and composition. A study was conducted in a target group of 56 *Fritillaria* species, based on the morphology of their nectaries and nectar composition to assess the significance of pollination mode as well as its predisposition for the evolution of bird pollination. All species studied had nectaries located at their tepal base and produced nectar, but their size, shape, color, and composition all varied. Most fritillaries had hexose-rich nectar, in easily accessible and unprotected nectaries. Scanning electron microscope (SEM) analysis revealed that the surface of the nectaries of most *Fritillaria* species was flat and clearly distinct from that of the surrounding tissues, which might be regarded as an adaptation for insect-pollination. Nectaries of *F. imperialis* were considerably larger and had dilute nectar without sucrose, which was produced profusely, thereby fulfilling the criteria characteristic of ornithophilous flowers. The copious nectar of presumed hummingbird-pollinated species was rather balanced and of medium sugar concentration. Their large lanceolate nectaries contrasted sharply with the tessellated background of their tepals. These characters might indicate a mixed pollination system that engages both birds and insects. Floral anatomy and microstructure and nectar composition for *Fritillaria* species in subgenera *Korolkowia* and *Liliorhiza* are studied for the first time.

Keywords: *Fritillaria*, nectar, pollinator shift, ornithogamy, nectary surface, SEM

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INTRODUCTION

The genus *Fritillaria* L. (type species *F. meleagris* L.) is a widely distributed member of Liliaceae (lily family). It comprises of 130 to 140 species occurring through most of the northern hemisphere (Tamura, 1998; Rønsted et al., 2005; Tomović et al., 2007; Mabberley, 2008; Day et al., 2014), with centers of speciation in the Mediterranean region, especially in Turkey and Greece (Rix, 1984;

Zaharof, 1986; Tekşen and Aytaç, 2011), Iran (Kiani et al., 2017), Western North America (Beetle, 1944; Rønsted et al., 2005), and East Asia (Hill, 2016). Recent phylogenetic analyses indicate that the genus may be paraphyletic with members of the subgenus *Liliorhiza*, principally North American species, forming a sister clade to the remaining *Fritillaria* species and the genus *Lilium* (Day et al., 2014).

Fritillaria are found in a variety of climatic regions and in different habitats, with about half of the genus occurring in locations categorized as open with dry summers (Rønsted et al., 2005). Fritillaries are located across a wide latitudinal range from coasts, riparian zones, meadows, woodland, steppe, deserts, mountain screes, and alpine zones (Xinqi and Mordak, 2000; Ness, 2003; Tekşen and Aytaç, 2011; Zych and Stpiczyńska, 2012; Rix and Strange, 2014).

Fritillaria species are bulbiferous, spring-flowering perennials with an erect flowering stem producing either a single flower or multi-flowered racemes. The flowers are usually actinomorphic and have a typical tulip-like, trimerous, campanulate perianth but with a nodding character (Tamura, 1998). They come in various colors such as white, pink, greenish, yellow, or purplish/reddish. The perianth parts of many species, including the type species, have a characteristic checkerboard pattern; hence, the name of the genus – fritillus, Latin for dicebox (Zych et al., 2014).

This astonishing floral diversity may have developed in response to their pollinator interactions, although, flower visitors have only been recorded for six species of *Fritillaria* (White, 1789; Hedström, 1983; Búrquez, 1989; Peters et al., 1995; Bernhardt, 1999; Minagi et al., 2005; Kawano et al., 2008; Zox and Gold, 2008; Zych and Stpiczyńska, 2012). These limited records are unlikely to reflect the complete spectrum of pollination vectors. Pollinator effectiveness has only been evaluated for bumblebee-pollinated *F. meleagris* (Zych and Stpiczyńska, 2012; Zych et al., 2013, 2014) and to a lesser extent the pollination of *F. imperialis* by birds and insects (Peters et al., 1995).

The first observation of a *Fritillaria* flower visitor was made by White (1789). He recorded small birds drinking nectar from *F. imperialis*. After 200 years, similar observations were also made for *F. imperialis* in other European gardens by Búrquez (1989) and Peters et al. (1995). Blue and great tits visited and efficiently pollinated flowers. Bumblebees were also seen visiting and pollinating the large and pendant flowers of *F. imperialis*, but pollinator efficiency was lower than bird visitations (Peters et al., 1995). Although there are no pollinator records for *F. imperialis* in the wild, it has been proposed that bird pollination is the most likely vector. This hypothesis is supported by the presence of a landing platform suitable for passerine birds and large pendant flowers with large volumes of diluted nectar (Peters et al., 1995). For some North American species (*F. gentneri* and *F. recurva*), hummingbird pollination has been recorded in natural habitats (Pendergrass and Robinson, 2005; Cronk and Ojeda, 2008), possibly attracted by their bright red flowers. The only *Fritillaria* species occurring in both Asia and North America, the typically dark-flowered *F. camschatcensis*, is habitually visited by flies (Zox and Gold, 2008). Flowers of this species emit a disagreeable smell like rotting flesh, and it might be described as a typical example of carrion fly-pollination syndrome (Willmer, 2011). There are only six fritillaries that have been noted to have a sweet scent: *F. liliacea* (King, 2001), *F. obliqua* (Beck, 1953), *F. striata* (Santana, 1984), *F. stribrnyi* (personal observation LH), *F. tortifolia* (personal observation LH), and *F. yuminensis* (Leon et al., 2009), all presumably visited by bee species. Bees have only been recorded to visit *F. ayakoana* (Minagi et al., 2005) and *F. meleagris* (Hedström, 1983; Stpiczyńska et al., 2012; Zych et al., 2014).

Generally, flower pollinators are attracted by a combination of visual and olfactory cues. Although the data is limited, one might assume this is true for *Fritillaria*. Floral scent, but to the best of our knowledge, it has only been explored in studies for *F. meleagris* (Hedström, 1983). In the study conducted by Zych and Stpiczyńska (2012), many bees collected *Fritillaria* pollen, but the most common floral food-reward appeared to be nectar. This was secreted by perigonal nectaries positioned adaxially on each of the six perianth segments (Stpiczyńska et al., 2012). Until now, the most comprehensive description of *Fritillaria* nectar diversity was given by Rix and Rast (1975), who studied nectar sugars in 37 European and Asiatic species of the genus. These authors found that nectar generally contained all three common nectar sugars, namely sucrose, glucose, and fructose. The only exception in their dataset was *F. imperialis* which produced no sucrose, confirming an earlier report by Wykes (1952). Rix and Rast (1975) concluded that nectar composition and, in particular, the fructose/glucose ratio may be an important diagnostic character in the infrageneric taxonomy of *Fritillaria*.

The position, morphology, and structure of nectar-secreting glands have been key taxonomic characters and have been investigated by many researchers, notably in Asiatic species by Bakhshi Khaniki and Persson (1997) and for *F. meleagris* by Stpiczyńska et al. (2012). For example, nectaries are lanceolate to linear in members of the subgenus *Fritillaria*, circular in the subgenus *Petilium* (L.) Baker, and in the subgenus *Rhinopetalum* (Fisch. ex Alexand) the nectaries are deeply depressed and situated in sac-like projections (Bakhshi Khaniki and Persson, 1997). This latter study, however, provides only general morphological descriptions and information concerning the ultrastructure of nectaries, is only available for *F. meleagris* (Stpiczyńska et al., 2012; Zych and Stpiczyńska, 2012). Studies of nectaries and nectar characteristics are important, not only from a taxonomic point of view but also with respect to ecological and evolutionary studies of the genus. For example, based on nectar characteristics, Rix and Rast (1975) posited that bees and wasps were the main pollinators of *Fritillaria*. In fact, floral visitors of *Fritillaria* flowers include insects of the orders Hymenoptera (mostly various species of bees and wasps), Diptera, Lepidoptera, and Coleoptera (Hedström, 1983; Bernhardt, 1999; Naruhashi et al., 2006; Kawano et al., 2008; Zych and Stpiczyńska, 2012; Zych et al., 2013). Although usually concealed by the perianth, in some *Fritillaria* species optically copious nectaries also play an important ecological role in guiding the pollinators to the nectar once they have been attracted to the flowers by other traits (tepal color, scent, etc.). This appears to be the case in ornithophilous *F. imperialis* (Cronk and Ojeda, 2008) and, perhaps, also in other bird-pollinated species. However, despite the diversity and wide distribution of the genus, these aspects of *Fritillaria* diversity are

still neglected to a great extent. In particular, detailed studies of the flower, and, especially the study of nectar-secreting structures and nectar composition have only been conducted for a very limited number of species. To date, the most complete analysis using light microscopy (LM) and scanning electron microscopy (SEM) was completed by Bakhshi Khaniki and Persson (1997) for members of four subgenera represented in Central Asia: *Fritillaria*, *Petilium* (L.) Baker, *Rhinopetalum* (Fisch. ex Alexand) Baker, and *Theresia* (K. Koch) Baker. However, no information is available for fritillaries from North America or the Far East. This paper represents the first study of floral anatomy and microstructure, as well as nectar composition, for a broad range of *Fritillaria* species, including, for the first time, taxa from two subgenera: *Korolkowia* Rix and *Liliorhiza* (Kellogg) Benth. & Hook.f. Our objectives were (1) to verify the presence of secretory tissues in selected members of the genus, (2) to investigate the microstructure of their nectaries, and (3) to compare nectar production and composition in the taxa studied in order to shed light on the evolution of their pollination systems.

MATERIALS AND METHODS

Plant material used for this study was obtained from *Fritillaria* species cultivated at the University of Warsaw Botanic Garden (BG), from the private collections of the coauthors (LH and PK) and from the private collection of Colin Everett (Somerton, Somerset, United Kingdom; CE). Many *Fritillaria* species are very rare in cultivation, and the number of specimens used for each type of analysis varied because of the availability of fresh plant material (accession numbers for species in this investigation are listed in **Table 1**).

Microscopical Observations

All microscopical examinations were conducted for flowers in full anthesis. Flowers from 1 to 10 were selected for morphometric measurements of the nectary size and position. If less than three specimens were available, all flowers from one individual were measured, and if more plants were available, the flowers studied were selected randomly. The study was conducted with the use of a digital caliper Borletti DIN 862 (Borletti, Italy), tethered to a computer to record the values. Shape, size, structure, and color of the nectaries were observed in the fresh material using a Nikon SMZ 1000 stereomicroscope (Nikon Corp., Japan).

SEM Observations

Three areas were selected for SEM observations on the outer tepals: the nectaries, the area distal to the nectaries, and the tip of the tepals. Sections of nectaries collected in the greenhouse and from PK collection were prepared by fixing nectary tissue in 2.5% glutaraldehyde in phosphate buffer (pH 7.4; 0.1 M). After three washes in phosphate buffer, they were postfixed in 2% (w/v) osmium tetroxide solution for 2 h and were dehydrated in a graded ethanol series. After dehydration, samples were subjected to critical point drying using liquid CO_2 and were sputter-coated with gold. Nectaries gathered from the collections of LH and CE were transported from the United Kingdom to Warsaw in 70% ethanol. Subsequently, the material was prepared for SEM as described above, and the sample was examined using a SEM LEO 1430VP (Zeiss, Germany) and Zeiss Libra 120 (Zeiss, Germany).

Seven representative species (*F. eduardii*, *F. gentneri*, *F. michailovskyi*, *F. persica*, *F. recurva*, *F. raddeana*, and *F. sewerzowii*), either characterized by visually different nectary structures or representing closely related species, were prepared as semi-thin nectary sections.

Plant material was prepared by fixing nectary tissue in 2.5% glutaraldehyde in phosphate buffer (pH 7.4; 0.1 M). The samples were then washed three times before postfixation in 2% (w/v) osmium tetroxide solution for 2 h and were dehydrated in a graded ethanol series. After dehydration, they were infiltrated with LR White resin. Succeeding polymerization at 60°C, the nectaries sections were cut with a glass knife. The semi-thin sections (0.9–1.0 μm thick), stained with an aqueous solution of 1% methylene blue/1% Azure II (1:1) for 5–7 min on a hot plate (60°C), were prepared for LM and analyzed for general histology.

Hand-cut sections of the nectaries of all studied species were also prepared for histochemical investigations by means of LM. The size of epidermal and parenchymal cells and the depth of nectariferous tissue were measured. Subsequently, hand-cut sections were stained with an alcoholic solution of Sudan IV for lipids and with Lugol's iodine solution for starch. Sections of nectaries were also stained with aniline blue and were examined by means of fluorescence microscopy (FM) in order to test for the presence of callose in cell walls.

Nectar Sampling

Flowers for nectar sampling in the collections of BG and PK were first selected during the bud stage and were bagged with nylon mesh (net 0.5 mm) to prevent visits by insects. During anthesis but before anther dehiscence, nectar was sampled. In the BG collection flowers, progress was checked daily in the morning and in the afternoon for the presence of nectar. Nectar sampling in the LH and CE collections was from unbagged flowers open to animal visitors. All nectar was sampled with microcapillary pipettes from nectaries of all six tepals and was combined as one sample per flower. In the case of *F. camschatcensis*, nectar volumes were very small and sampling with microcapillaries pipettes was performed under a Nikon SMZ 1000 stereomicroscope (Nikon Corp., Japan). The collected nectar was subsequently expelled from microcapillaries onto a refractometer prism RL-4 (PZO, Poland) in order to measure nectar sugar concentration.

Nectar was also sampled to assess nectar sugar composition. Nectar from a standing crop of unbagged flowers was collected for this purpose. Particular care was taken during nectar collection, to avoid any contamination by pollen, phloem soap, or any other plant tissue. However, as most of the *Fritillaria* have downward facing flowers the risk of pollen contamination was low.

No attempts were made to emasculate flowers prior to sampling, so that sugar composition represents nectar as encountered by visitors. Nectar from one to three flowers of each species was placed into 1.5 ml Eppendorf tubes prior to analysis using high performance liquid chromatography (HPLC). The samples were frozen (−20°C) until required. Nectar was diluted

TABLE 1 | Investigated species of the subgenus *Fritillaria*.

	MIC	SEM	NEC	DIS	VOL	CON	NECTAR SUGAR PROFILE						SOU
							FRU	GLU	SUC	MAL	LAC	T	
F. acmopetala	+	+	24.2 ± 2.8 (86)	3.8 ± 0.6 (86)	34.8 ± 17.8 (42)	35.4 ± 15.7 (41)	76	23	1			132.2 (3)	>10 BG, LH, PK
F. amana	+	+	14.4 ± 2.2 (34)	4.7 ± 0.9 (29)	7.8 ± 2.3 (5)	47.8 ± 13.5 (5)	38	1	61			411.2 (1)	>10 BG, LH
F. armena	+	+	2.6 ± 0.9* (6)	0 (6)*	42.6 ± 19.4 (4)	14.1 ± 11.2 (3)							6 BG
F. aurea	+	+	5.35 ± 0.5 (10)	6.5 ± 2.8 (10)		31 (1)							2 PK
F. bithynica	+		12.1 ± 0.8 (11)	1.2 ± 1.1* (6)			47	52	1			572.7 (1)	2 BG, PK
F. carica	+	+	2.6 ± 0.4 (36)	0.7 ± 0.1 (36)	13.6 ± 5.8 (14)	21.1 ± 12.7 (46)	47	44	7	2	?	344 (2)	>10 BG, PK
F. caucasica	+		10.5 ± 2.1 (18)	0.4 ± 0 (18)	1.4 ± 0.7 (3)	26.2 ± 11.9 (6)							5 BG,PK
F. conica	+		10.2 ± 11.6* (6)	1.8 ± 0.6* (6)		66.2 ± 3.4 (3)	30	30	40			310.82 (1)	3 BG
F. crassifolia	+	+	26.4 ± 1.1* (6)	2.7 ± 0.5* (6)			25	28	47			117.72 (1)	2 LH, PK
F. davisii	+	+	11.7 ± 3.4 (22)	3.2 ± 0.3* (17)									5 BG
F. elwesii	+	+	10.5 ± 1.7* (6)	0.8 ± 0.1* (6)	51 (1)	44 (1)	43	17	40			234.6 (2)	1 BG
F. graeca	+	+	5 ± 0.6 (6)	1.2 ± 0.2* (6)	9.6 ± 1 (11)	52.6 ± 10.7 (17)	45	40	15			217.4 (1)	>10 BG. PK. LH
F. gussichiae	+	+	23 ± 1.9 (6)	8.5 ± 0.5* (6)	44.5 ± 9 (4)$^{+}$	14.5 ± 3.6 (4)							4 PK
F. involucrata	+	+	33.5 ± 4.3* (6)	4.3 ± 0.7* (6)		17 (1)	13		87			88.8 (1)	1 LH
F. kotschyana	+		13.9 ± 1.3 (24)	5.4 ± 0.8 (6)	55.3 ± 8.1 (4)	24.3 ± 7.5 (6)	73	27				133.9 (1)	>5 LH. PK
F. latakiensis	+	+	11.6 ± 1.1 (24)	1.2 ± 0.3 (24)		37.6 ± 7.1 (4)							4 LH
F. lusitanica	+		20.7 ± 5* (6)	3.3 ± 0.5* (6)	51^{+}	9	27	3	70			156.5 (1)	1 LH
F. meleagroides	+	+	19.3 ± 3.5* (6)	4.7 ± 0.7* (6)	39.1 ± 7.7 (9)	31.6 ± 13.4 (11)	59	15	26			246.1 (1)	>10 LH. PK
F. michailovskyi	+	+	14.9 ± 5.1 (67)	3.6 ± 0.6 (34)	3.6 ± 3.5 (18)	17.4 ± 8.3 (8)	47	19	34			163.1 (3)	>10 BG
F. minuta	+	+	2.4 ± 0.5* (6)	1.4 ± 0 (6)	1.2 ± 1.4 (11)	64.9 ± 11.4 (13)							>10 BG. PK
F. montana	+		25.9 ± 2.7* (6)	4 ± 0.2* (6)	1.3 (1)	14.5 (1)							3 BG. PK.LH
F. mutabilis	+	+	12.2 ± 3.2* (6)	2.2 ± 0.5* (6)									3 LH
F. obliqua	+		2.5 ± 0.6* (6)	0.9 ± 0.2* (6)	2.8 (1)	61 (1)							1 PK
F. olivieri	+		12.5 ± 0.4 (24)	2 ± 0.4 (24)	11.6 ± 3.2 (4)	35.7 ± 8.1 (5)							>5 BG, LH, PK
F. pallidiflora	+	+	4.1 ± 1.0 (44)	5.4 ± 0.7 (35)	25.9 ± 18.2 (69)	43.7 ± 17.1 (70)	43	46	21			341.6 (1)	>10 BG
F. pinardii	+	+	2.3 ± 0.4* (6)	0.1 ± 0.0* (6)		40 (1)	49	47	1	3		282.2 (1)	3 BG. LH, PK
F. pontica	+	+	6.8 ± 0.8 (24)	3.2 ± 0.3 (24)	5.6 ± 5.8 (6)	16.05 ± 3.3 (6)	58	30	10			92.6 (2)	>5 BG
F. pyrenaica	+	+	15 ± 1* (6)	4.9 ± 0.7* (6)	52.3 ± 13 (6)	12.8 ± 4.5	37	14	49			200.4 (2)	>5 LH. PK
F. ruthenica	+		13.6 ± 0.9 (6)	2.4 ± 0.3* (6)	0.1 (1)								>5 BG. LH
F. sibthorpiana	+	+	3.8 ± 0.7* (6)	0.8 ± 0.2* (6)									1 BG

(Continued)

TABLE 1 | Continued

	MIC	SEM	NEC	DIS	VOL	CON	NECTAR SUGAR PROFILE						SOU
							FRU	GLU	SUC	MAL	LAC	T	
F. stribrnyi	+	+	4.7 ± 0.8* (6)	0.8 ± 0.0* (6)	7 (1)	35 (1)							1 BG
F. thessala	+	+	13 ± 2.3 (24)	5.10.3 (24)	14 (1)	16.2 ± 0.2 (3)	30	4	66			133.9 (2)	<5 BG. LH. PK
F. thunbergii	+		5.9 ± 0.9* (6)	1.1 ± 0.2* (6)									2 BG, PK
F. tubiformis	+	+	4.4 ± 0.3* (6)	5.7 ± 4.6* (6)									1 PK
F. ussuriensis	+		21 ± 3* (6)	1.6 ± 0.4* (6)	3.20 (1)	77.5 (1)	23	2	75			377.2 (1)	1 BG
F. uva-vulpis	+	+	11.9 ± 1.7 (32)	0.02 ± 0 (20)	12 ± 13.7 (25)	47.8 ± 17.1 (28)	37	41	22			241.4 (1)	BG. LH. PK
F. verticillata	+	+	4.8 ± 0.3 (4)	2.6* (4)	0.6^{+} (1)		48	52				781.8 (1)	1 LH
F. whittallii	+	+	14.6 ± 2.6 (28)	1.3 ± 0.1 (27)	13.5 ± 3.8 (4)	50.9 ± 7.3 (4)	46	13	41			405.8 (2)	5 BG. PK
Subgenus *Japonica*													
F. amabilis	+	+	5.9 ± 0.3* (6)	0.8 ± 0.2* (6)			44	24	32			491.8 (1)	1 LH
F. ayakoana	+	+	5.9 ± 0.4* (6)	2 ± 0.4* (6)	0.9^{+} ± 0.7 (3)	40.7 ± 0.5 (3)							3 LH
Subgenus *Korolkowia*													
F. sewerzowii	+	+	11.8 ± 3.9 (34)	0 (34)	24.6 ± 17.5 (33)	61.9 ± 11.7 (35)	50	50				280.6 (1)	>10 BG
Subgenus *Liliorhiza*													
F. affinis	+		26.9 ± 5.5* (6)	4.7 ± 1* (6)	15.4	12	58	38	4			379.2 (1)	1 OB
F. camschatcensis	+	+	4.6 ± 0.7 (30)	0 (30)			58	40	2			41.3 (1)	>10 BG
F. eastwoodiae	+	+	2.5 ± 0.6* (6)	0.2 ± 0* (6)	33.8^{+} ± 7.2 (3)	16.3 ± 2.5 (3)	65	32	3			151.55 (1)	1 CE
F. gentneri	+	+	10.8 ± 3.4 (14)	2.2 ± 0.3 (14)	54 ± 9.8 (10)	31.1 ± 18.1 (8)	50	30	20			525.85 (2)	>5 BG,CE
F. liliacea	+	+	2.8 ± 0.6* (6)	0.4 ± 0.1* (6)	34^{+} (1)	48 (1)							1 CE
F. recurva	+	+	5. ± 0.2 (24)	1.3 ± 0.9 (24)	49.2 ± 18.3 (25)	33.1 ± 10.6 (25)	58	33	9			163.1 (1)	>5 BG. CE
Subgenus *Petilium*													
F. eduardii	+	+	21.4 ± 1.4 (13)	2.6 ± 0.3 (13)	56.5 ± 18.3 (17)	5 ± 8.1 (24)	80	20				17.86	>10 BG, PK
F. imperialis	+	+	29.6 ± 1.8 (46)	1.5 ± 0.1 (46)	204.8 ± 94.7 (28)	13.6 ± 3.7 (20)	49	51	0			68	>10 BG
F. raddeana	+	+	2.8 ± 2.2 (38)	4.3 ± 0.5 (38)	8.7 ± 1.4 (8)	50.1 ± 15.7 (31)	43	30	52			227.7 (2)	>10 BG
Subgenus *Rhinopetalum*													
F. bucharica	+		10.5 ± 0.7 (36)	0.8 ± 0 (36)	0.3 ± 0 (4)	52.7 ± 1.8 (5)							>10 BG
F. gibbosa	+	+	7.7 ± 0.8* (6)	0.1 ± 0* (6)									4 PK
F. stenanthera	+	+	11.6 ± 1.4 (30)	0.2 ± 0 (30)	0.6 ± 0.8 (25)	45.5 ± 15.9 (27)	32	63	5			218.7 (1)	>10 BG
Subgenus *Theresia*													
F. persica	+	+	3.5 ± 0.4 (64)	2 ± 0.3 (54)	4.3 ± 4.5 (74)	46.5 ± 18.7 (69)						825.8 (1)	>10 BG
Other species													
F. grandiflora	+	+	14.7 ± 1* (6)	6.4 ± 0.6* (6)	42.3 ± 1.3 (2)	23.8 ± 2.8 (2)							2 PK
F. olgae	+		19.6 ± 1.9* (6)	2.5 ± 0.2* (6)	74.4 ± 33.2 (5)	29.5 ± 14 (5)	37	2	61			199.4 (1)	5 BG

Investigated species of subgenera Japonica (F. amabilis and F. ayakoana), Korolkowia (F. sewerzowii), Liliorhiza (F. affinis, F. camschatcensis, F. eastwoodiae, F. gentneri, F. liliacea, F. recurva), Petilium (F. eduardii, F. imperialis, and F. raddeana), Rhinopetalum (F. bucharica, F. gibbosa, and F. stenanthera), Theresia (F. persica) and other species. MIC, microscopical analysis; staining with Lugol's iodine solution and Sudan IV; SEM, scanning electron microscope; NEC, nectary size; DIS, distance from nectary to the base of the perianth, data presented as means (number of petals studied) ± SD referring to technical and biological replicates or only to technical replicates (marked with); VOL, nectar volume (when measured only from standing crop marked with $^{+}$); CON, nectar concentration, data presented as means (number of inflorescences studied) ± SD; Nectar sugar profile [%]: FRU, fructose; GLU, glucose; SUC, sucrose; MAL, maltose; LAC, lactose; T, total amount of sugars [mg/ml]; SOU, source and number of specimens obtained.*

with water to a volume of 50 μl (10 μl of nectar + 40 μl of water). The sample was filtered through spin columns using a 0.4 μm pore size membrane filter before injection. The supernatant was then loaded into the insert. An Agilent 1260 Infinity Series HPLC system with autoinjector, refrigerated autosampler compartment, thermostatted column compartment, quaternary pump with in-line vacuum degasser, and refractive index detector was used. A ZORBAX Carbohydrate Analysis Column (4.6 mm × 250 mm, 5 μm) was used for sugar separation and analysis. A 10 μl aliquot sample or standard solution was injected. The separation was conducted at 30°C with the mobile phase comprising acetonitrile:water (70:30, v/v) at a flow rate of 1.4 ml/min. The analytical data were integrated using the Agilent OpenLab CDS ChemStation software for liquid chromatography (LC) systems. Identification of sugars was performed by comparing retention times of individual sugars in the reference vs. test solution. The content of glucose, fructose, sucrose, maltose, and lactose was assayed based on comparisons of peak areas obtained for the samples investigated with those of the reference solutions.

RESULTS

Nectary Location and Structure, Nectar Secretion, Concentration, and Composition

In all species, six nectaries were located at the base of the tepals (**Figures 1**, **2**). The mean distance from the base of the perianth for all species studied was 2.3 ± 2.1 mm (means calculated only for technical replicates, if only one specimen was available, or means resulting from technical replicates were used to represent each biological replicate; missing SD values represent a single accession), in the range of 0.0 to 8.5 mm (**Table 1**). In all but one studied species, the nectaries of both outer and inner tepals were equally accessible to potential pollinators. Only in *F. persica* were the nectaries of the outer tepals not visible.

Nectary cells were smaller, flatter, and more regular in shape than other epidermal cells (not shown). In each case, the nectaries consisted of a single-layered epidermis (without stomata) and several layers of subepidermal parenchyma (**Figures 3D,E**)[1]. The cytoplasm of epidermal cells contained a large nucleus, small vacuoles, and plastids. Plastids were also present in deeper layers of the nectaries' parenchyma. Vascular bundles contained both xylem and phloem elements. Subepidermal nectary parenchyma consisted of 2–5 layers (**Figure 3F**). Staining with Lugol's iodine solution revealed no starch grains, with the exception of members of the subgenus *Petilium*, where staining revealed the presence of numerous starch grains in the plastids of epidermal and subepidermal cells (**Figure 4B**).

Staining with Sudan IV revealed the presence of numerous droplets of lipid on the epidermis (**Figure 3C**) and within nectary cells of all studied species, the cuticule on the surface of secretory epidermis stained red. Staining with aniline blue did not reveal the presence of callose in cell walls (**Figure 3D**). The mean area occupied by the nectaries of all studied species was 11.8 ± 8.6 mm^2, in the range of 1 to 38.2 mm^2 (**Table 1**).

In all of the investigated species, each of the six nectaries located adaxially on perianth segments produced nectar. Nectar passed across the cell wall and was exuded through pores in the cuticle.

The amount of nectar produced depended largely based on the species. On average, *Fritillaria* flowers produced 30.6 ± 52.2 μl of nectar, in the range of 0.4 to 204.8 μl (means and SDs; N = 498, 41 species; data pooled for all seasons and species investigated). The concentration of nectar, on average, was 39.1 ± 20.63%, in the range of 5 to 77.5% (N = 599, 44 species). Nectar in most species was hexose-rich (with mean total concentration of 278.6 ± 187.8 mg/ml). The sugar profile of nectar was dominated by sucrose and glucose, which were also detected in the nectar of all species (115.4 ± 77.2 mg/ml and 88.5 ± 122.2 mg/ml, respectively; N = 54, 34 species). Fructose was also a significant component of *Fritillaria* nectar (87.6 ± 84.4 mg/ml), but it was not present in the nectar of all species studied. Traces of maltose and lactose were also detected in the nectar of several species (4 ± 1.8 and 5.1 mg/ml, respectively) (**Table 1** and **Supplementary Material**).

Subgenus *Fritillaria*

This subgenus was represented by 38 species (**Table 1**). The nectaries of this subgenus were highly variable, and the nectaries differed greatly in size and area occupied (mean value 12.4 ± 8.8 mm^2). The average distance of the nectaries from the base of the perianth was 2.7 ± 2.2 mm. Several species had ovate nectaries of uniform background color (i.e., of poor contrast) and were difficult to differentiate. For example, *F. amana* had round nectaries, sometimes encircled by a brownish band, especially those of the inner tepals, but the nectaries were generally of a uniform green color. *Fritillaria aurea*, *F. bithynica*, *F. conica*, and *F. sibthorpiana* had round, slightly depressed, yellow or slightly greenish nectaries, similar in color to the rest of the tepal. *Fritillaria davisii* and *F. pyrenaica* (**Figure 5A**) had linear-lanceolate darker nectaries that did not contrast well against the tessellated brownish background. *Fritillaria elwesii* had ovate to triangular, greenish nectaries, of a slightly darker hue when compared to that of surrounding tissues. *Fritillaria pallidiflora* had triangular nectaries, greenish to yellowish and of the same color as the tepals.

Fritillaria uva-vulpis had ovate yellowish nectaries and a similarly colored background.

Considerably, more members of the subgenus *Fritillaria* had contrasting nectaries. *Fritillaria acmopetala*, *F. graeca*, *F. involucrata*, *F. latakiensis*, *F. mutabilis*, *F. olivieri*, *F. pontica*, *F. thessala*, and *F. verticillata* had ovate to obovate, dark nectaries that contrasted strongly against the brighter tepals. *Fritillaria kotschyana* and *F. whittallii* also had ovate to obovate, dark nectaries that contrasted strongly against the tessellated tepals. *Fritillaria minuta* had ovate, slightly depressed nectaries of dark green color and contrasting yellowish tepals. Similarly, *F. pinardii* and *F. carica*, had linear-lanceolate, deeply depressed, greenish nectaries, that were only slightly darker than the green-yellow tepals. Sometimes, there was slightly

[1]The photographs of flowers and nectaries are representative species for the *Fritillaria* subgenera.

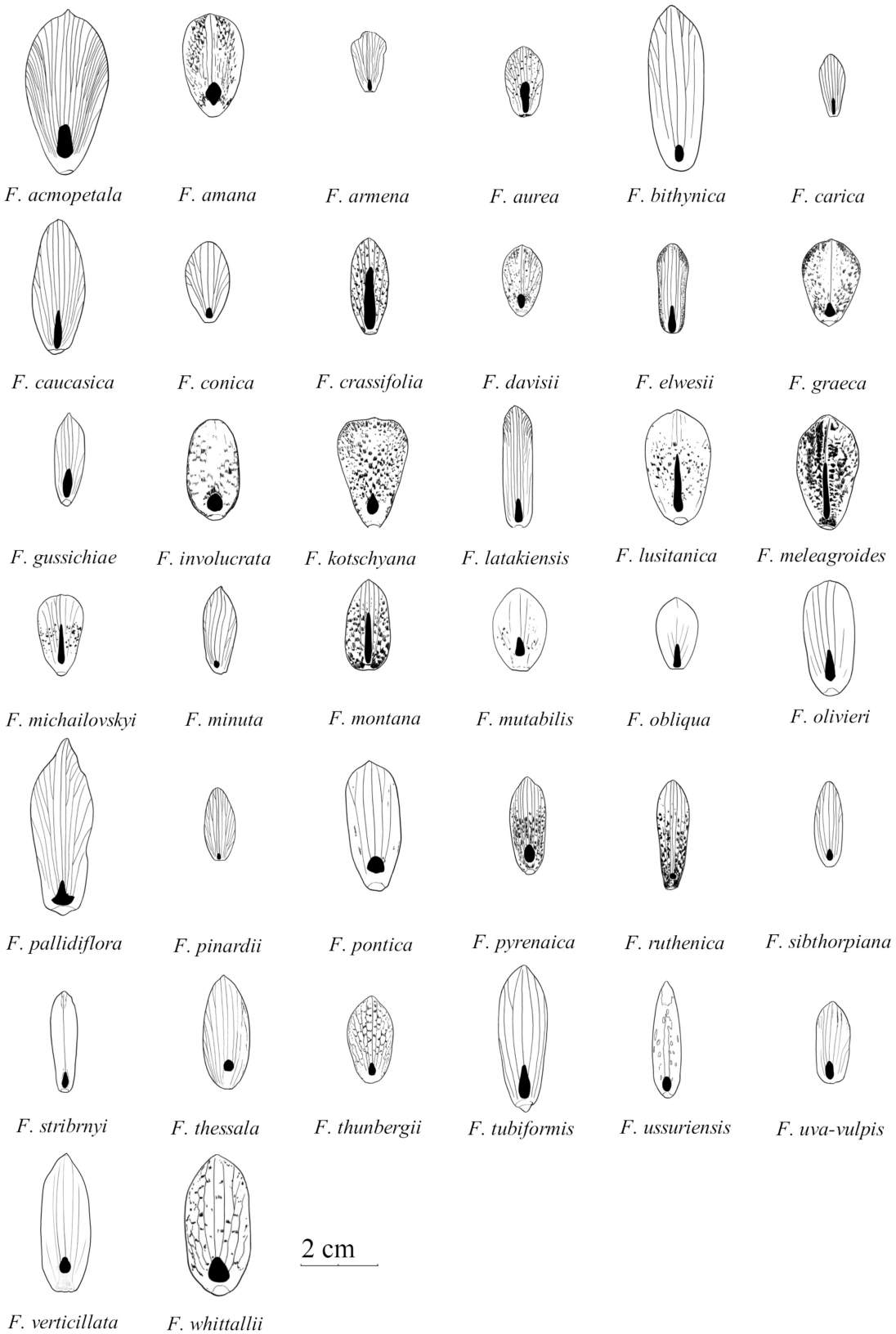

FIGURE 1 | Schematic drawings of outer tepals and nectaries (filled with black) in species studied of the subgenus *Fritillaria*. Size graded according to natural size of studied tepals (Drawn by Jan Kryciński).

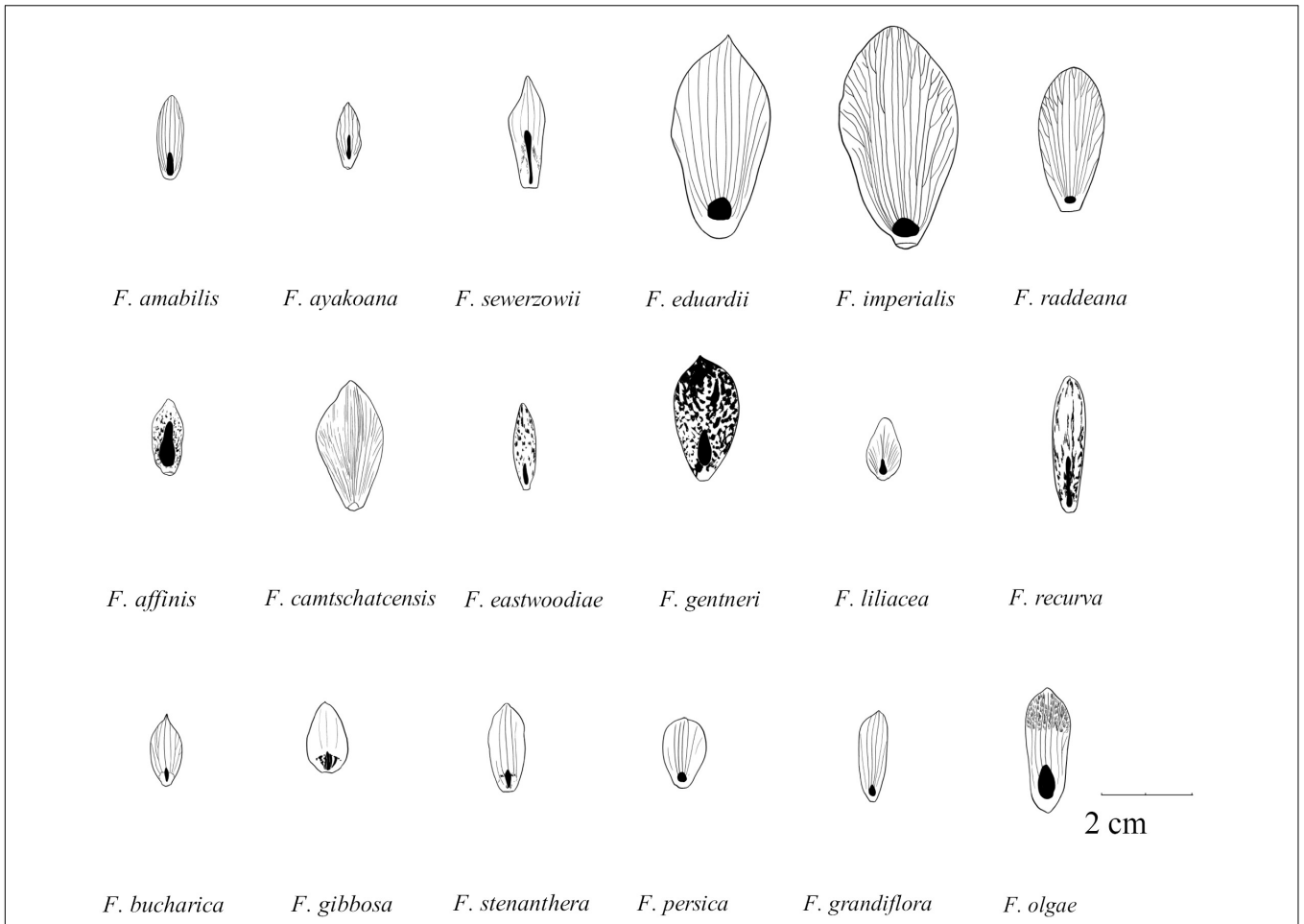

FIGURE 2 | Schematic drawings of outer tepals and nectaries (filled with black) in selected species of subgenera: *Japonica* (*F. amabilis and F. ayakoana*); *Petilium* (*F. eduardii, F. imperialis, and F. raddeana*); *Liliorhiza* (*F. affinis, F. camschatcensis, F. eastwoodiae, F. gentneri, F. liliacea, and F. recurva*); *Rhinopetalum* (*F. bucharica, F. gibbosa, and F. stenanthera*); *Korolkowia* (*F. sewerzowii*); *Theresia* (*F. persica*); and other species (*F. olgae and F. tubiformis*) (Drown by Jan Kryciński).

more contrast when the tepals were yellow. *Fritillaria lusitanica*, *F. ussuriensis*, *F. ruthenica*, and *F. michailovskyi* (**Figure 3A**) had greenish, oblanceolate nectaries surrounded by reddish, tessellated tepals. *Fritillaria thunbergii* had greenish, oblanceolate nectaries surrounded by yellowish, tessellated tepals. *Fritillaria caucasica*, *F. obliqua*, and *F stribrnyi* had linear-lanceolate, bright green nectaries contrasting with dark red tepals, like those of *F. montana*, where the green, slightly depressed nectaries were surrounded by red, tessellated tepals. *Fritillaria gussichiae* had ovate, bright green nectaries that contrasted strongly against a dark red background. *Fritillaria crassifolia* had linear nectaries that were usually green and heavily marked with purple. Nectaries were visible but did not contrast strongly against the green, red-tessellated tepals. Similarly, in *F. meleagroides*, the dark, linear nectaries were surrounded by green tepals with dark red tessellation. *Fritillaria armena* had nectaries at the base of the perianth. Several species had nectaries close to the base of the perianth (not more than 1 mm distant) or more than 5 mm from it, but for most of the species nectaries arose more than 1 mm but less than 5 mm from the base of the perianth (**Table 1**).

Scanning electron microscope analysis revealed that in most of the species investigated, the internal surface of the nectary was flat, while the surrounding area and the rest of the tepal was slightly undulate, owing to the slightly convex cells (**Figure 3B**). Nectaries of *F. verticillata* also had slightly convex cells. In *F. armena*, the area of the nectary was also surrounded by a row of elevated, rounded protrusions, also present on the tepals, where they were arranged in rows. In *F. davisii*, the remainder of the tepal was covered with elevated protrusions. *Fritillaria uva-vulpis* and *F. michailovskyi* had rows of elevated cells directly above the nectaries. In *F. pyrenaica* (**Figure 5B**), the area of the nectary was comprised of conical papillae. In *F. tubiformis*, nectary cells had papillae and the epidermal cells of the surrounding area were also slightly convex.

Plants of this subgenus produced variable amounts of nectar (32.3 ± 54.4 μl) of highly variable concentration (38.5 ± 20.6%.) The lowest mean concentration was recorded for *F. pyrenaica* (12.8 ± 4.5%, 52.3 ± 13 μl). The highest mean value was observed for *F. ussuriensis* (77.5%, 3.2 μl). The highest production was recorded for *F. kotschyana* (55.3 ± 8.1 μl,

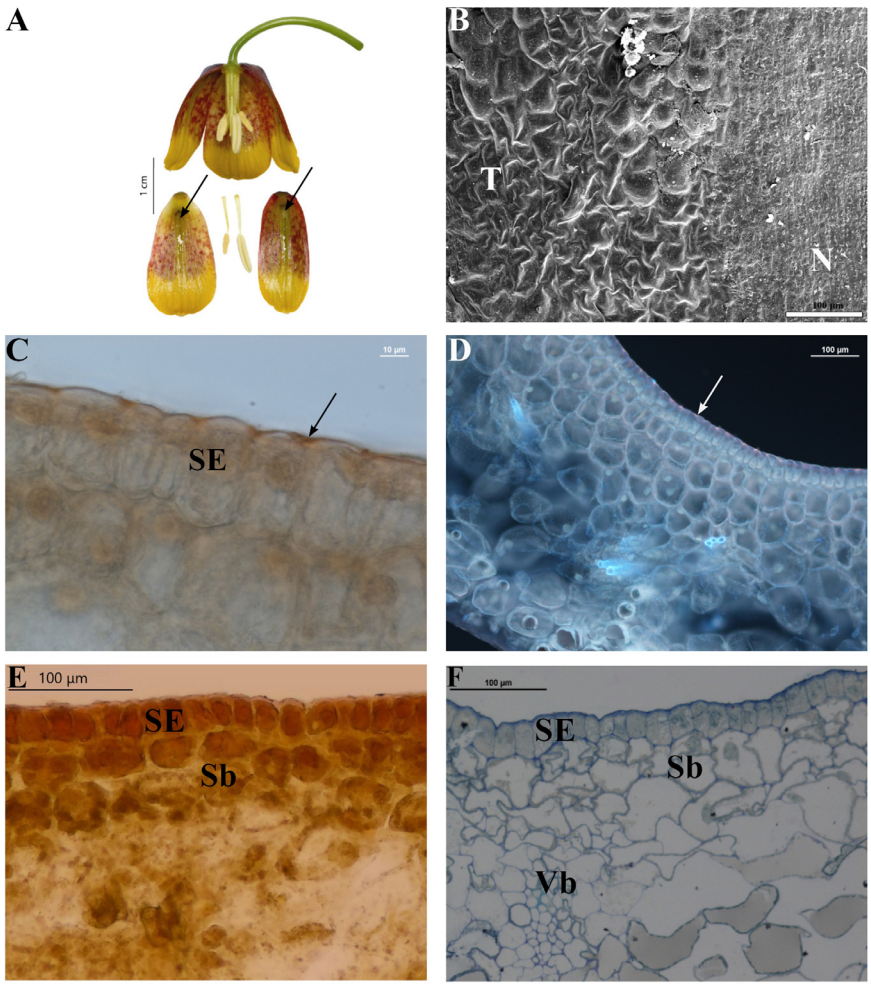

FIGURE 3 | Flowers and nectaries of *F. michailovskyi* at full anthesis. Macro, SEM, and LM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing flat nectary cells (N) and slightly convex cells of surrounding tepals area (T). **(C)** Cuticule on the surface of secretory epidermis (SE) of outer tepals stained with Sudan IV, arrow indicates lipids layer on the surface of cuticule of nectary. **(D)** Staining with aniline blue does not reveal the presence of callose in the walls of nectary cells. Arrow indicates lipids on the cuticle of the nectary of outer tepals. **(E)** Treatment with Lugol's iodine solution reveals the absence of starch in secretory (SE) and subsecretory parenchyma (Sb). Secretory epidermis with dense cytoplasm. **(F)** Staining with Azure II showing secretory epidermis (SE), subsecretory parenchyma (Sb), and ground parenchyma with vascular bundle (Vb).

24.3 ± 7.5%). The smallest volume was recorded for *F. verticillata* (0.6 µl, but this was too small to measure sugar concentration) (**Table 1**).

Subgenus *Japonica*

This subgenus was represented by two species (**Table 1**). Nectaries of these species were yellowish and ovate-lanceolate. In the case of *F. ayakoana* (**Figure 6A**), the base of the nectary was green and it contrasted strongly with the bright tepals. In the middle of the nectary, there were small upwardly curved ridges or protuberances. The area occupied by nectaries of the members of this subgenus varied little and, on average, measured 5.4 ± 1 mm^2. The nectaries were placed close to the base of the perianth (**Table 1**).

Scanning electron microscope analysis revealed that the surface of the nectaries and the surrounding areas of *F. ayakoana* (**Figures 6B,C**) flowers were identical with conical projections (**Figures 6B,D**).

The layer of subepidermal nectary parenchyma was deeper in *F. ayakoana* and was four or more cells deep, whereas in *F. amabilis*, it was 2–3 cells deep.

During anthesis, the entire nectary area was coated with nectar (no data regarding nectar replenishing available). Flowers of the species studied produced, on average, 0.9 ± 0.7 µl nectar of concentration 40.7 ± 0.5% (**Table 1**).

Subgenus *Korolkowia*

This is a monotypic subgenus containing *F. sewerzowii*. Its nectaries were long, elliptical, and depressed in a groove (**Figure 7A**), which was surrounded by a row of longitudinal papillose ridges (**Figure 7B**). They did not cover the nectaries, which were clearly visible, green, and were strongly contrasting

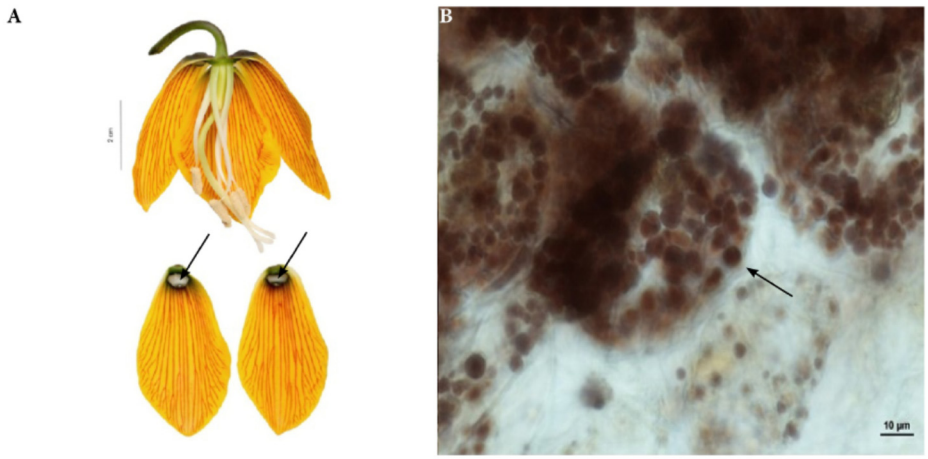

FIGURE 4 | Flowers and nectaries of *F. imperialis* at full anthesis. Macro and LM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Treatment with Lugol's iodine solution reveals the presence of starch in secretory and subsecretory parenchyma. Grains visible in the plastids of subepidermal cells in *F. imperialis* outer tepal nectaries.

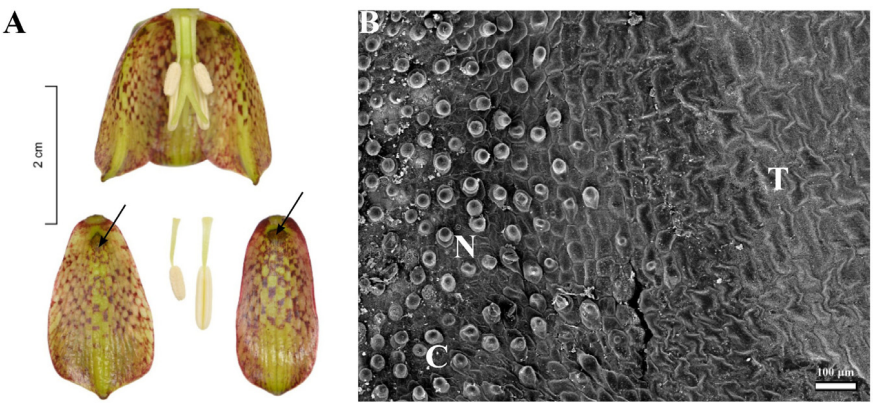

FIGURE 5 | Flowers and nectaries of *F. pyrenaica*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing the nectary area (N), comprising conical papillae and slightly convex cells (C) of surrounding tepals area (T).

with the dark background. The area of the nectaries was measured to be 11.8 ± 3.9 mm^2, and the nectaries were located at the base of the perianth (**Table 1**).

Scanning electron microscope analysis revealed that the surface of the nectaries was slightly undulate and wrinkled. The cells of the surrounding area were slightly convex (**Figure 7B**).

During anthesis, the entire nectary was coated with nectar, which was replenished on its removal. Flowers of this species produced, on average, 24.6 ± 17.5 µl nectar of concentration 61.9 ± 11.7% (**Table 1**).

Subgenus *Liliorhiza*

This subgenus was represented by six species (**Table 1**). Nectaries of *F. camschatcensis* were very narrow, lanceolate and were hidden in the ridges (**Figure 8C**). Its surface was covered with proturbance and it glistened; therefore, the nectaries always looked as if they contained nectar. The nectaries of other *Liliorhiza* species were ovate-lanceolate in shape and were not protected by any additional structures. Nectaries of *F. affinis*, *F. recurva*, *F. gentneri*, and *F. eastwoodiae* were similar in appearance and were brightly colored against a contrasting darker, tessellated background (**Figure 9A**). The nectaries of *F. liliacea* (**Figure 8A**), like the surrounding part of the tepal, were uniformly green and, thus, almost invisible. *Fritillaria affinis* had the largest nectaries (29.6 ± 5.5 mm^2), and the smallest nectaries were recorded for *F. liliacea* (2.8 ± 0.6 mm^2). The nectaries were generally situated close to the base of the perianth (0.9 ± 0.9 mm) (**Figure 9A** and **Table 1**).

Scanning electron microscope analysis revealed that the surface of the depressed nectaries of *F. eastwoodiae* consisted of slightly convex epidermal cells, as well as in the surrounding area. *Fritillaria liliacea* and *F. camschatcensis* also had depressed nectaries surrounded by a row of elevated cells having a grooved

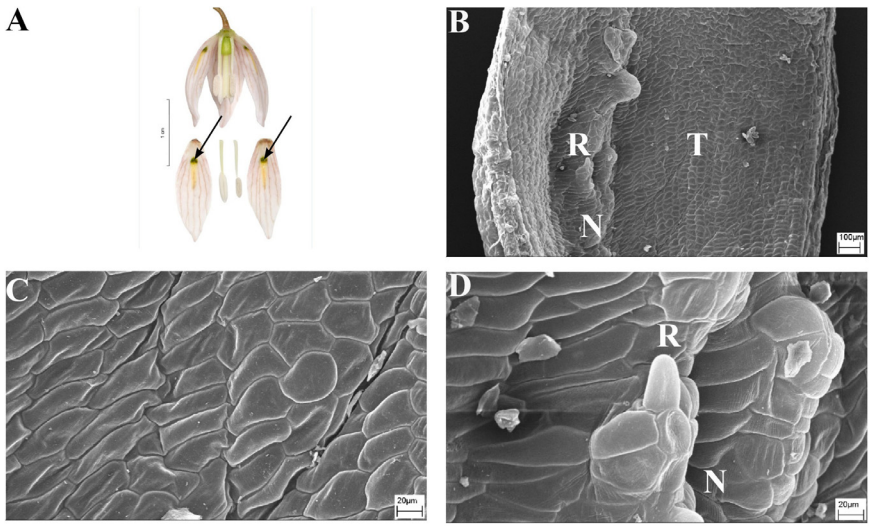

FIGURE 6 | Flowers and nectaries of *F. ayakoana*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing the nectar-bearing area (N) with upwardly curved ridge or protuberance on its surface and slightly convex surrounding area (T). **(C)** Slightly convex cells of outer tepal surface directly above the nectary. **(D)** Cuticule of the nectary (N) of outer tepal with protuberance (R).

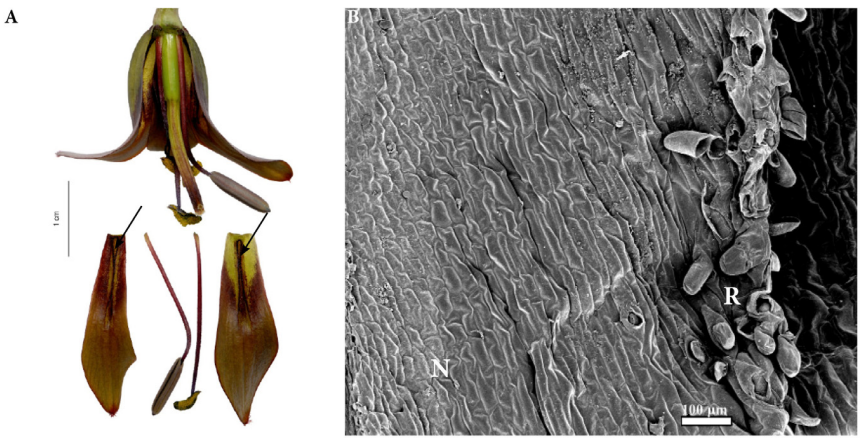

FIGURE 7 | Flowers and nectaries of *F. sewerzowii*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing the nectary (N), surrounded by papillose ridges (R).

surface (**Figures 8B,D**). Both *F. gentneri* and *F. recurva* had depressed nectaries surrounded by elevated cells (**Figure 9B**).

The subepidermal nectary parenchyma consisted of four or more layers. Only in *F. eastwoodiae* was the nectary parenchyma shallower and consisted of 2–3 layers.

In *F. gentneri* and *F. recurva*, nectar was replenished on its removal. *Fritillaria camschatcensis* produced very small amount of barely visible, viscous nectar. Owing to the consistency of the nectar and the fact that the nectary surface was glistening, it was not possible to assess nectar replenishment. No data was available for *F. eastwoodiae* and *F. liliacea*. Plants of this subgenus produced copious amounts of nectar (48 ± 17.1 µl) of average concentration 30.1 ± 11.9%. The lowest concentration of nectar was recorded for flowers of *F. affinis* (12%), and the highest was recorded for *F. liliacea* (48%). The smallest volume of nectar was produced by *F. affinis* (15.4 µl), and the greatest volume of nectar was produced by *F. gentneri* (54 ± 9.8 µl) (**Table 1**).

Subgenus *Petilium*

This subgenus was represented by three species (**Table 1**). The nectaries were depressed, elliptic, or round in shape. In *F. imperialis* and *F. eduardii*, they were similar in size with an area of 27.7 ± 4.7 mm^2 and were similarly located 1.7 ± 0.7 mm above the base of the perianth. Nectaries of *F. raddeana* (**Figure 10A**) were smaller (2.8 ± 2.2 mm^2) and were located 4.3 ± 0.5 mm above the base of the tepal (**Table 1**). The white nectaries of *F. imperialis* (**Figure 4A**) and *F. eduardii* contrasted sharply with the surrounding dark green background. Nectaries of *F. raddeana* were not as strongly contrasting as were those of the two previous

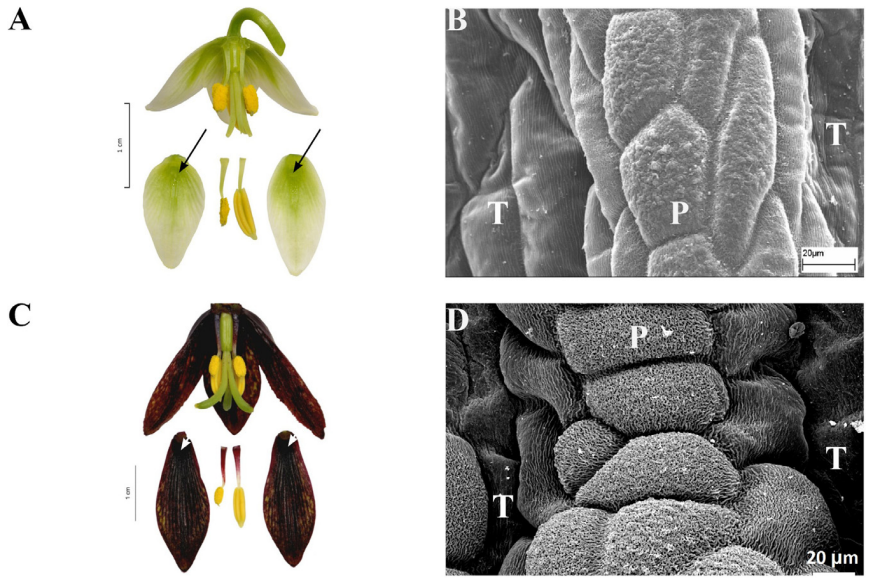

FIGURE 8 | Flowers and nectaries of *F. liliacea* **(A,B)** and of *F. camschatcensis* **(C,D)**, both in full anthesis. **(A)** Flowers and tepals of *F. liliacea*, nectaries marked with arrows. **(B)** Protrusions surrounding the nectary area (P) on *F. liliacea* outer tepal (T). **(C)** Flowers and tepals of *F. camtschatcensis*, nectaries marked with arrows. **(D)** Protrusions surrounding the nectary area on the outer tepal of *F. camschatcensis*.

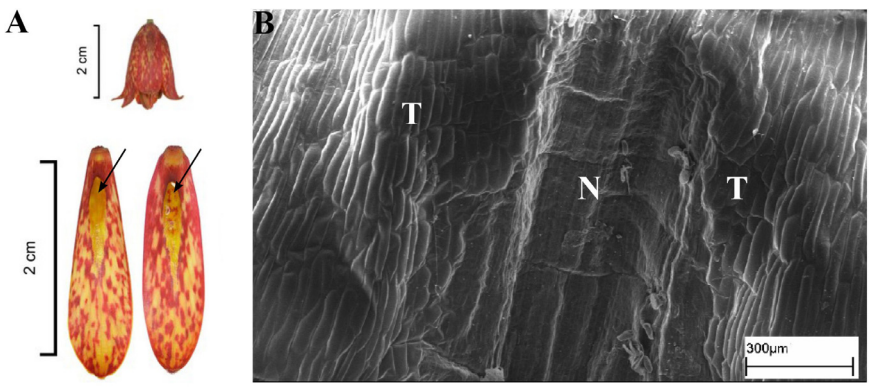

FIGURE 9 | Flowers and nectaries of *F. gentneri*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing deeply depressed nectary (N).

species described; they were darker and surrounded by a similarly dark background.

Scanning electron microscope analysis revealed that the inner surface of nectaries of *F. imperialis* and *F. eduardii* were flat, whereas the surrounding area and the rest of the tepals were slightly undulate. In *F. raddeana*, the area within the nectary was similar to the remainder of the tepal area and was also slightly undulate (**Figure 10B**). Subepidermal nectary parenchyma consisted of 2–4 layers. Staining with Lugol's iodine solution revealed the presence of numerous starch grains in the plastids of epidermal and subepidermal cells (**Figure 4B**).

During anthesis, the entire nectary area was coated with nectar. It was easily accessible in the form of large droplets. Nectar was replenished on its removal. Flowers of this subgenus produced, on average, 133.3 ± 107.5 µl of nectar of concentration 26.2 ± 23.1%. The highest concentration and the smallest volume were recorded for *F. raddeana* (50.1 ± 15.7% and 8.7 ± 1.4 µl, respectively). The lowest concentration was recorded for *F. eduardii* (5 ± 8.1%). Flowers of *F. imperialis* produced the largest volume of nectar recorded for the subgenus *Petilium* (204.8 ± 94.7 µl) (**Table 1**).

Subgenus *Rhinopetalum*

This subgenus was represented by three species (**Table 1**). The typical nectaries were deeply depressed spurs, bearing two densely papillose ridges (**Figure 11A**). The ridges were adpressed, protecting access to the nectaries. In *F. stenanthera*, there were two additional papillose ridges adjacent to the nectary area (**Figure 11B**). Nectaries were visible on the reverse side of the tepals as dark "horns." These projections differed from

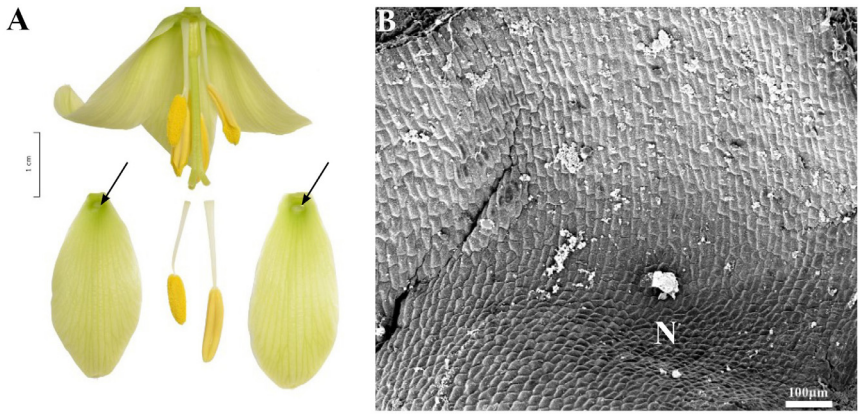

FIGURE 10 | Flowers and nectaries of *F. raddeana*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing depressed, cup-shaped nectary (N), and slightly convex cells of surrounding area.

FIGURE 11 | Flowers and nectaries of *F. stenanthera*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing deeply depressed spurs nectary (N), protected by densely papillose ridges (R).

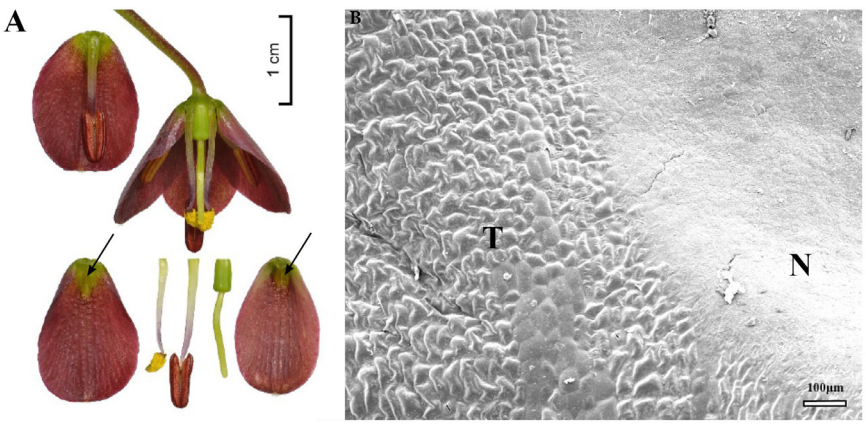

FIGURE 12 | Flowers and nectaries of *F. persica*. Macro and SEM images. **(A)** Flowers and tepals, nectaries marked with arrows. **(B)** Part of outer tepal showing the flat nectary area (N) and slightly convex cells of surrounding area (T).

species to species; in *F. gibbosa*, one of the tepal "horns" was always significantly larger than the other. In *F. stenanthera*, all projections were of the same size. In *F. bucharica*, they were of similar size – more prominent at the bud stage, becoming flatter in mature flowers. In this species, nectaries were uniformly green, like the background, but the nectaries at the top were darkly spotted, located just above the entrance to the nectary. In *F. gibbosa*, the area surrounding the nectaries was dark brown, but the ridges were paler. Nectaries of *F. stenanthera* had both green and brown elements (**Figure 11A**). The surrounding area was mostly brown, and the sides were greenish or dark yellow. The deeply depressed area within the spur of the nectaries of *Rhinopetalum* was densely clothed with short cilia. Nectaries were flat and glabrous (**Figure 11B**).

It was difficult to measure the size of nectaries for representatives of *Rhinopetalum*, since, they were hidden inside the spur, and the borders of the nectary were not obvious. Moreover, in *F. gibbosa*, one of the nectaries was significantly larger. The approximate mean size of the nectary for members of this genus was 7.9 ± 3.6 mm^2. The largest nectaries were recorded for *F. stenanthera* (11.6 ± 1.4 mm^2), and the smallest nectaries were recorded for *F. gibbosa* (7.7 ± 0.8 mm^2). Nectaries of all species were located very close to the base of the perianth, the mean value of the distance to the perianth being 0.6 ± 0.3 mm (**Table 1**).

Scanning electron microscope analysis revealed that the surface consisted of three distinct parts. The area of nectar secretion was represented by a depressed groove having a flat surface. It was surrounded by slightly elevated walls and had a slightly undulate surface. The rest of the spur was densely coated with short cilia. The remainder of the tepal was slightly undulated (**Figure 11B**).

Subepidermal nectary parenchyma consisted of four or more layers, and the nectar-bearing area occupied a relatively narrow region located at the center of the tepals.

During anthesis, the entire nectary area was coated with nectar, although it was not visible and was protected by the papillose ridges. Nectar was replenished on its removal. Flowers of this subgenus produced, on average, 0.5 ± 0.8 μl of nectar of concentration 46.6 ± 14.8%. On average, *F. bucharica* produced 0.3 ± 0 μl of nectar of concentration 52.7 ± 1.8%, with *F. stenanthera* producing 0.6 ± 0.8 μl and having the lower concentration of 45.5 ± 15.9% (**Table 1**).

Subgenus *Theresia*

The nectaries of *F. persica* were slightly depressed and triangular in shape. They occupied an area measuring 3.5 ± 0.4 mm^2 and were located 2 ± 0.3 mm above the base of the perianth (**Figure 12A** and **Table 1**). The green nectaries contrasted sharply with the surrounding dark purple background. However, several nectary cells were pigmented.

Scanning electron microscope analysis revealed that the inner surface of nectaries was flat, whereas the surrounding area and the remainder of the tepal bore slightly convex cells (**Figure 12B**). Subepidermal nectary parenchyma consisted of 3–5 layers.

During anthesis, the entire nectary area was coated with nectar, which extended beyond the nectary. Nectar from the inner tepals was easily accessible and lacked projections for protection, but nectar from the outer tepals was concealed behind the inner tepals. Nectar was replenished on its removal.

Fritillaria persica produced, on average, 4.3 ± 4.5 μl of nectar of concentration of 46.5 ± 18.7% (**Table 1**).

Other Species

We also studied the nectaries of *F. grandiflora* and *F. olgae*, which are not classified into any subgenus (Rix, 2001). However, both of them presumably belong to the subgenus *Fritillaria* (Rix, 1974; Kiani et al., 2017). *Fritillaria grandiflora* had darkly colored, round nectaries, surrounded by tessellated tepals. *Fritillaria olgae* had ovate to triangular, darkly colored nectaries that contrasted with the green tepals. Nectaries of *F. grandiflora* measured 14.7 ± 1 mm^2 and were located 6.4 ± 0.6 mm from the base of the perianth, for F. olgae, these values were 19.6 ± 1.9 mm^2 and 2.5 ± 0.2 mm, respectively (**Table 1**).

Scanning electron microscope analysis revealed that the inner surface of the nectary was flat, whereas the cells of the surrounding area and the remainder of the tepal were slightly convex. Subepidermal nectary parenchyma consisted of 3–5 layers.

During anthesis, the entire nectary area was coated with nectar. Unless collected, large droplets of nectar were found at the edges of tepals in *F. olgae*. Nectar was replenished on its removal. *Fritillaria grandiflora* produced, on average, 42.3 ± 1.3 μl nectar of concentration 23.8 ± 2.8%. *Fritillaria olgae* produced, on average, 74.4 ± 33.2 μl of concentration 29.5 ± 14% (**Table 1**).

DISCUSSION

The present study reports SEM and LM analyses and descriptions of the nectaries for 56 species of *Fritillaria* contained in seven subgenera, including 29 species which were studied for the first time. This study is also the first to examine nectary surface of members of subgenera *Japonica*, *Korolkowia*, *Liliorhiza*, and *Theresia*. The most likely area used as a landing site for insect pollinators was imaged under SEM.

We also conducted SEM studies of the relevant area, which might be considered as the probable landing site for insect pollinators.

The nectaries of *Fritillaria* are positioned adaxially on each of the six perianth segments (**Figures 1**, **2**). However, several other nectary features, such as size, shape, or color, were generally variable among species. Such variation in shape and position of the nectaries was previously described by Rix and Rast (1975), Bakhshi Khaniki and Persson (1997), and Kiani et al. (2017), who studied the nectaries of 31 taxa from four subgenera using SEM and LM.

Despite differences in the appearance of nectaries, their morphology and positon were similar within the different subgenera. However, our studies echoed the conclusions of Bakhshi Khaniki and Persson (1997) that morphology and position of nectaries might be important diagnostic features in the taxonomy of the genus, as the nectaries of different subgenera vary greatly. In contrast, nectary ultrastructure, which is similar

among all the species studied, does not provide any taxonomic information. The only exception is the subgenus *Petilium*, which is distinguished from other subgenera by the presence of starch in nectary cells.

We examined the nectaries for the occurrence of callose, which may push the projections into the cytoplasm and facilitate deposition of wall material (Offler et al., 2003), but we did not observe it in the cell walls of any species, although it was previously detected as the component of wall ingrowths in *F. meleagris* by Stpiczyńska et al. (2012). This difference might be species-specific (*F. meleagris* was not included in this study) or dependent on the flowers' development stage. Clearly, this needs further studies. However, the outer epidermal cells and/or nectary cells in all species contained lipid droplets. The presence of a lipid layer on the plant surface may provide a way to reduce water loss. However, the role of lipid within nectary cells is in need of investigation (Kamińska and Stpiczyńska, 2011).

The present study also provides information about nectar sugar composition for 34 species and the quality and volume of this reward for additional 46 species. Since the nectar properties, like concentration and amount available for floral visitors, are highly variable, it cannot serve as a taxonomic tool. Also, contrary to Rix and Rast (1975), our study indicates that the fructose/glucose ratio varies greatly within and between the species studied; therefore, it does not provide useful taxonomic information.

Subgenus *Fritillaria*

Usually, nectaries are more or less flat, surrounded by an area with slightly convex cells. Only in *F. pyrenaica* did we find the area around the nectary to be comprised of dense conical papillae, a feature that had not been previously described for any other *Fritillaria* species. In several species like *F. davisii*, *F. uva-vulpis*, and *F. michailovskyi*, there are 'warts' on the tepals or on the border of the nectary (Bakhshi Khaniki and Persson, 1997).

Rix and Rast (1975) studied nectar properties for several members of the subgenus *Fritillaria*. We obtained similar results for *F. bithynica*, *F. elwesii*, and *F. pyrenaica*. In comparison with the other species studied by Rix and Rast (1975), *F. pallidiflora, F. crassifolia, F. michailovskyi, F. acmopetala*, and *F. pontica* had glucose-dominant nectar in this study. *Fritillaria amana* produced sucrose-dominant nectar (H:S in the ratio of 2:3). Differences in the results obtained might be related to the method used (gas liquid chromatography vs. HPLC). However, it is evident that many complex factors affect nectar properties such as time of collection, weather conditions while sampling, or variation in the nectar properties depending on the development of the inflorescence (Willmer, 2011).

Subgenus *Japonica*

Scanning electron microscope analyses and studies of nectar properties were conducted for the first time for species in the subgenus *Japonica*. The structure of the nectary and tepal surface was similar to that of other *Fritillaria* species studied. However, in the middle of the nectar-bearing area of *F. ayakoana* (**Figures 6B,D**) was a small, upwardly curved ridge or protuberance, a feature previously described for *F. kaiensis* and *F. japonica* (Naruhashi et al., 1997). Similar ridges also occur in the closely related subgenus *Rhinopetalum* (Bakhshi Khaniki and Persson, 1997). We conducted the first nectar sugar analysis for *F. amabilis* (the volume of *F. ayakoana* nectar was too small to be collected in the field). It was similar to that found in other fritillary species investigated, that is, hexose-dominant, with a relatively high sugar concentration (41%). However, the amount of nectar produced was considerably lower than the mean value for the genus, and again it was similar to the values detected in members of the closely related subgenus *Rhinopetalum*.

Subgenus *Korolkowia*

Scanning electron microscope analyses and studies of nectar properties were conducted for the first time for *F. sewerzowii*. The tips of *F. sewerzowii* nectaries are visible from outside the flower; however, the main part is concealed within the very narrow, bell-shaped perianth (**Figure 7A**).

The surrounding papillose ridges only partly restrict access to the nectaries, in contrast to the ridges present in the members of *Rhinopetalum*, which almost completely cover the nectary. In this study, nectary area and floral reward were studied for the first time. The nectary area, unlike that of most fritillaries, is not flat, but it is slightly undulate and wrinkled (**Figure 7B**). Surprisingly, flowers of this species produced nectar without any trace of sucrose, a result which was previously only described for *F. imperialis* (Rix and Rast, 1975).

Subgenus *Liliorhiza*

Nectaries and nectar in this subgenus were studied for the first time. SEM analysis revealed that the nectaries of *F. liliacea* (**Figure 8B**) were surrounded by a row of elevated cells with grooved surfaces, similar to those found in *F. camschatcensis* (**Figure 8D**). Flowers of most members of this subgenus produced copious nectar (mean value 48–49 μl) of medium sugar concentration (mean value 31%); only in the flowers of *F. camschatcensis* were traces of viscous nectar found. Nectar was hexose-dominant, although, the nectar of *F. gentneri* also contained a substantial amount of sucrose (20%).

Subgenus *Petilium*

In this study, the nectaries of the subgenus *Petilium* differed from the other species studied. They are elliptical (Bakhshi Khaniki and Persson, 1997) and depressed. Anatomical studies revealed large accumulations of starch (**Figure 4B**), which was not previously reported for *Fritillaria* nectaries. Large amounts of starch, found, for example, in bee-pollinated *Anemopaegma album* (Bignoniaceae), are thought to be responsible for the secretion of large amounts of sugar during the peak secretory period (Dafni and Vereecken, 2016; Guimaraes et al., 2016). Furthermore, the white, glistening appearance of nectaries within the subgenus *Petilium* may also result from the presence of starch; since, the flat upper epidermis may act as a thin film reflector responsible for its glossiness. It may further serve as a filter to backscattered light as the starch bodies located in the parenchyma layers have strong light-scattering properties, as described for *Ranunculus* spp. (van der Kooi et al., 2017). Moreover, the

convex-shaped nectaries always appear full of nectar, even when they are empty.

An earlier study (Rix and Rast, 1975) considered *F. imperialis* to be distinct within *Fritillaria*, as sucrose was absent from its nectar. This study also found that sucrose was absence in the nectar of *F. eduardii*. In contrast, the nectar of the very closely related *F. raddeana* contains sucrose and can be described as balanced, based on the ratios of the sugars it contains (fructose, glucose, and sucrose in the ratio 4:3:5). There are large differences in the rate of nectar production within the subgenus *Petilium*. Both *F. imperialis* and *F. eduardii* produce large volumes (204 μl per flower) of dilute nectar (9%), whereas *F. raddeana* produces small volumes (7 μl per flower) of highly concentrated nectar (51%). Theoretically, nectar volumes are under strong selection pressures (e.g., balancing the costs and benefits of nectar production to the plant) and genetic control. However, differences in the volume/sugar concentration of even closely related species have previously been published (Davis et al., 1994).

Subgenus *Rhinopetalum*

As previously described, the nectaries of this subgenus are furrowed or lobed (Bakhshi Khaniki and Persson, 1997). The aperture of the nectary spur is densely surrounded with short papillae. However, the surface of the nectary area is flat and smooth (Bakhshi Khaniki and Persson, 1997). The presence of the papillae may protect the small quantity of nectar from evaporation or crystallization (Nicolson and Thornburg, 2007). Floral features are influence by ecological factors, like habitat type (Petanidou et al., 2006), and the members of *Rhinopetalum* are normally found in more arid habitats, like semideserts, than is normal within *Fritillaria* (Rix and Zarrei, 2007a,b; Kiani et al., 2017). This study provides the first record of a hexose-rich species (*F. stenanthera*) within the subgenus.

Subgenus *Theresia*

In both this study and that of Bakhshi Khaniki and Persson (1997), nectaries of *F. persica* were bright and green and contrasted strongly against a dark background (**Figure 12A**). However, Kiani et al. (2017) showed that the appearance of the nectary area as distinct depends on the flower color which is highly variable (pale green, pale yellow, bright yellow, orange, or dark purple), and in some populations, the nectaries of *F. persica* may be difficult to differentiate. Nectaries of the outer tepals are shielded by the inner tepals and are, therefore, probably not easily accessible to visiting insects. SEM analysis revealed that like those of most *Fritillaria* species, the nectaries were uniform and flat and surrounded by an area occupied by slightly convex cells of the tepals (**Figure 12B**). Flowers of *F. persica* produce rather a small amount of nectar, but as a single specimen usually produces several dozen flowers, the overall reward is relatively plentiful. Nectar is strongly hexose-dominant.

Other Species

Scanning electron microscope analyses and studies of nectar properties were conducted for the first time for *F. grandiflora* and *F. olgae*. The nectaries of *F. grandiflora* and *F. olgae* were similar to those found in flowers of the subgenus *Fritillaria*. Also, SEM analysis revealed the typical *Fritillaria* pattern, comprising a flat and uniform nectary area surrounded by an area bearing convex cells. Both these features might indicate affinities to the subgenus *Fritillaria*. However, *F. olgae* nectar was sucrose-dominant (H:S in the ratio 2:3), such as it is generally found in passerine-pollinated species in the subgenus *Petilium*.

Ecological Context

The flowers of *Fritillaria* are very diverse – not only in color, shape, and appearance but also in the array of floral rewards like nectar sugar concentration and composition or reward location (Bakhshi Khaniki and Persson, 1997). *Fritillaria* have a wide geographical distribution and occupy a variety of different habitats (Hanson et al., 2009; Kiani et al., 2017). Recent DNA studies show a strong geographic relationship within *Fritillaria* (Day et al., 2014), even among morphologically divergent species. Species rich areas are normally associated with highly variable habitats and/or more recent oscillating climates and microclimates, resulting in numerous range changes, periods of isolation, and recombination (Myers et al., 2000; Kiani et al., 2017). However, some elements of this remarkable diversity might also be the result of a relatively rapid coevolution with their pollinators, as several species, which are distantly related have similar-looking nectaries (convergence): like, for example, *F. pudica* and *F. carica* or *F. purdyi* and *F. crassifolia*, respectively (Rix and Strange, 2014). As many fritillaries are native to remote, difficult to access, or uninhabited areas (Kiani et al., 2017), information regarding their reproduction is limited. Data concerning pollination system or *Fritillaria* flower visitors are only available for six species (Hedström, 1983; Búrquez, 1989; Peters et al., 1995; Minagi et al., 2005; Pendergrass and Robinson, 2005; Zox and Gold, 2008; Zych and Stpiczyńska, 2012; Zych et al., 2014).

In temperate habitats of the northern hemisphere, where *Fritillaria* species grow, most plants are insect-pollinated and are characterized by lack of specialization of their flowers, thus, attracting a large range of insects (Galetto et al., 1998). This is generally the case for *Fritillaria*, where the nectaries are variable and most are easily accessible, therefore, are likely to be visited by a range of different floral visitors. Our microscopical studies revealed that the structures of nectaries of putatively insect pollinated species are similar. Most *Fritillaria* species studied had a relatively flat nectary area surrounded by slightly convex cells, important for insect pollination, providing extra perch during flower manipulation by insects, thus, increasing foraging efficiency (Whitney et al., 2011; Ojeda et al., 2012). Conical papillae, found on the nectaries of *F. pyrenaica*, cause the thin film of the nectar to glisten. In *F. davisii*, the tepal surface and the area adjacent to the nectary was covered with papillae, arranged in rows along the length of the tepal. This may act as a physical nectar guide and a tactile cue, orientating insects toward both the reward and the reproductive parts of the flower. As the floral reward is easily accessible, and the corolla is normally wide open, insects can easily locate and exploit this resource.

Although data from the literature is scare, bees were seen by authors, visiting *Fritillaria* flowers. These animals frequently

seek out flowers with medium nectar volumes of medium sugar concentration, often located toward the base of the flower (Willmer, 2011), criteria common in *Fritillaria* flowers. Many *Fritillaria* species have hexose-rich nectar, which according to floral syndrome theory is preferred by short-tongue bees (Chalcoff et al., 2006). However, bee-pollinated plants show a wide range of nectar sugar compositions, as would be expected in the nectar of flowers pollinated by such a large group (Stiles and Freeman, 1993). Pollination by bees is the most common pollinating interaction, and it would be fair to expect that melittophily is the most common syndrome in *Fritillaria*.

Other types of entomogamy are also present in *Fritillaria*, for example, *F. camschatcensis* is fly-pollinated (Zox and Gold, 2008). The checkered pattern found on many flowers of *Fritillaria* might encourage increased visitation by carrion-flies or wasps, with a strong preference for mottled petals. These groups of animals often visit large, tubular flowers with wide-open corollas and dull red, purple, brown, or greenish petals (Willmer, 2011). Several other *Fritillaria* species, such as *F. graeca*, *F. montana*, and *F. davisii* fall into this category. These species produce relatively small volumes of nectar and sometimes emit a disagreeable odor. In *F. camschatcensis* and *F. davisii*, traces of viscous and almost solid nectar form a thick film over the nectary. This would be difficult for pollinators to access. Such presentation of nectar may act as a phenotypic filter, preventing insects other than flies, with have a cushion-like labium, to gather floral rewards (Stpiczyńska et al., 2014).

To date, there is no data on pollinators or floral visitors to members of the subgenus *Rhinopetalum*. They have unusual nectaries concealed in sac-like structures, covered with trichomes, which are not easily accessible. Densely papillose ridges of the nectary apertures potentially exclude feeding animals with relatively short proboscises (Stolar and Davis, 2010) and/or reduce evaporation. All three species examined in this subgenus, produce small volumes of nectar with relatively high sugar concentration. Pale pink or white flowers and nectar concealed in grooves covered with fine hairs are the normal characteristics associated with butterfly pollination, which also occurs in *Lilium martagon*, another species with similar nectaries (Brantjes and Bos, 1980).

Pollinator availability is low for winter or early spring flowering plants as low temperatures impede insect pollinator activity. By contrast, birds, which might be considered 'alternative pollinators,' are warm-blooded and more reliable at low temperatures, especially where cold and/or rainy weather conditions might be frequent (Fang et al., 2012). Although several studies indicate that frequent pollinator shifts have occurred during angiosperm speciation events, it may be the case that a large proportion of these events occur relatively late within specific pollination systems (Serrano-Serrano et al., 2017). This might also be the case for *Fritillaria*. Moreover, evidence indicates that the switch from entomophily to ornithophily occurred at least twice during the history of the genus, once for each of the two main clades.

Two very closely related Asian members of the subgenus *Petilium*, *F. imperialis* and *F. eduardii*, fulfill many of the criteria that characterize ornithophilous flowers. They show diurnal anthesis, have scarlet or orange flowers, and lack nectar guides. Their pale anthers and style extend beyond the large corolla, and these robust reproductive elements are able to withstand visits by large pollinators. Although birds do not display innate preference for red (Bené, 1945; Stiles, 1976; Micheneau et al., 2006; Handelman and Kohn, 2014), flowers that are visited by these animals often have red colouration (Goldsmith and Goldsmith, 1979; McDade, 1983; Delph and Lively, 1989). This might suggest that some new characters in bird-pollinated flowers have evolved to discourage visits by illegitimate flower visitors, in this instance insects (Cronk and Ojeda, 2008; Lunau et al., 2011). In *F. imperialis* and *F. eduardii*, the pollen is pale, which makes it less attractive to insects and less prone to potential pollen theft (Wilmsen et al., 2017).

Analysis of nectary morphology revealed the absence of collenchyma, this could have helped the flower to withstand contact with a hard beak, as it occurs in several ornithophilous flowers (Stpiczyńska et al., 2004, 2005, 2009). The starch grains, found in all members of the subgenus *Petilium*, might be regarded as a derived strategy to support the intensive secretion of large amounts of sugar during peak nectary activity (De la Barrera and Nobel, 2004; Heil, 2011; Stpiczyńska et al., 2012). Our studies reveal that this kind of energy storing in members of *Petilium* had two possible results. *Fritillaria raddeana* produces small volumes of highly concentrated nectar, whereas *F. eduardii* and *F. imperialis*, on the other hand, produce large volumes of very dilute nectar. In fact, in *F. eduardii*, the concentration of nectar sugar does not even reach 10%. The results for these two species match the data available in the literature, which state that bird-pollinated flowers produce nectar whose low sugar concentration averages 20–25% (w/w) (Nicolson, 2002). This indicates that the attraction of potential bird-pollinators might be important to the various nectar features related to pollination. Moreover, the nectar of *F. imperialis* and *F. eduardii* is hexose-rich, and it lacks even traces of sucrose. As nectar originates from sucrose-rich phloem sap, the proportion of monosaccharides in the final nectar depends on the activity of invertases in the nectary wall. Hydrolysis of sucrose increases the osmolality of the nectar, and the resulting water influx can convert a 30% sucrose nectar into a 20% hexose nectar with a great (1.6 fold) increase in volume. As passerine birds are the largest bird pollinators, they require large amounts of energy and water (Nicolson, 2002).

Different components of nectar respond in different ways to various environmental factors like elevation. Relative sucrose concentration declines in response to increasing elevation, but the percentage of fructose intensifies (Stiles and Freeman, 1993). Usually, this process is gradual, suggesting the response is physiological, possibly temperature related, rather than a reduction in the selection of sucrose-rich nectar. *Fritillaria imperialis* grows on rocky slopes at about 1000–3000 m (Tekşen and Aytaç, 2008; Kiani et al., 2017), whereas *F. eduardii* grows at 1200–2100 m and *F. raddeana* grows at 1000 m (Kiani et al., 2017), and the average sucrose concentrations reflect this; the decline in the case of *Petilium* is not gradual. Moreover, species of certain plant families have nectars of relatively consistent sucrose composition (Willmer, 2011), which is also not reflected for *Petilium*. The concentration and composition of nectar varies

greatly within this subgenus (*F. imperialis* and *F. eduardii* vs. *F. raddeana*). Nevertheless, the higher hexose content in the nectar of highland plants might originally have facilitated the switch to nectarivory by passerine birds (Stiles, 1978; Stiles and Freeman, 1993), and this may play a significant role in members of *Petilium*. Physiological constraints related to nectar production at higher altitudes may have led to sucrose elimination. Both *F. eduardii* and *F. imperialis* have pendulous, orange or reddish flowers, held on top of a thick stem, which provides a suitable perch for foraging birds. This might potentially lead to further pressure to reduce nectar concentration, which makes flowers less attractive to insect visitors and more attractive to birds, indicating that the nectar properties of *F. imperialis* and *F. eduardii* are the result of double selective pressure.

A second shift to ornithophily occurred in the distinct branch, consisting of mostly American species. Two species, sometimes co-occurring *F. recurva* and *F. gentneri*, also fulfill many of the criteria characteristic of ornithophilous flowers, that is, diurnal anthesis, scarlet flowers lacking nectar guides, and production of copious amounts of rather dilute nectar. However, the flowers of both species are held on a thin, pendulous inflorescence not suitable for perching while feeding but would suit hummingbird pollination (Willmer, 2011). The stamens of *F. recurva* are extended beyond the corolla tube, and, thus, allow contact between the reproductive elements and larger flower visitors. *Fritillaria gentneri* is a naturally occurring hybrid between *F. affinis* and *F. recurva*, and it possesses many intermediate flower features, but, without extended stamens. Flowers of *F. gentneri* and *F. recurva* are also visited under natural conditions by andrenids and halictids (Pendergrass and Robinson, 2005). It is likely that they are pollinated both by insects and birds, and we did not find many characters that might discourage illegitimate visitors. Moreover, *F. affinis* is postulated as an insect pollinated species.

Scanning electron microscope analysis revealed that the flat nectary area is surrounded by an area comprising slightly convex tepals cells. This might provide tactile cues for insect pollinators. Bees, for example, prefer such a surface for landing (Whitney et al., 2011). It might also help them to maintain their grip and stay inside the flowers while obtaining nectar from the flat nectary area. The epidermal wall was no thicker than in other species, nor more collenchymatous; it did not seem to provide any extra support and/or protective function, such as preventing damage to the nectary area while coming into contact with the hard beaks (Stpiczyńska et al., 2004, 2005, 2009).

As previously mentioned, nectar of *F. gentneri* and *F. recurva* was more copious and of lower concentration when compared to other closely related species of the subgenus *Liliorhiza*. However, the relative proportion of sugars is similar for all members of the subgenus *Liliorhiza* studied here, and it is characterized by high hexose concentration. Also, nectar of hummingbird-pollinated species is hexose-rich, which is unusual for hummingbird-pollinated flowers. Generally, hummingbirds visit flowers that have sucrose-dominant nectar (Cronk and Ojeda, 2008), also this matches the birds' recorded preferences in taste tests (Baker, 1975). The intestinal walls of hummingbirds contain a sucrase enzyme, which helps them to tolerate sucrose-rich solutions (del Rio and Karasov, 1990). However, data on hummingbird preferences are often conflicted (del Rio and Karasov, 1990; Lotz and Nicolson, 1996; Willmer, 2011), as these birds freely take hexose-rich nectar when other sources are unavailable (Willmer, 2011). Many flowers visited by hummingbirds are not distinctly adapted to hummingbird-pollination. However, the capacity of hummingbirds to easily extract nectar from open melittophilous flowers, may account for the many shifts toward ornithophily. Moreover, hummingbirds are inquisitive and they investigate many flower types and designs (Wilson et al., 2007), and their spatial memory helps them to return to rewarding plants (Healy and Hurly, 2003). Plants can benefit from these visits, as hummingbirds efficiently transfer pollen even with flowers of a poor morphological fit (Wilson et al., 2007).

Characters found in putative insect-pollinated species of *Fritillaria*, such as rapid nectar replenishment and large, brightly colored corollas, may be regarded as preadaptations for bird-pollination (Wilson et al., 2007). This is, especially, evident in *F. olgae*, a species that produces copious, but rather dilute, sucrose-rich nectar (Castellanos et al., 2003).

Flower features determine which animals or group of animals will be attracted. Moreover, the character and location of the reward can significantly influence the species that are attracted. The relationship between the characteristics of *Fritillaria* nectar and nectaries and their diversity may guide two evolutionary processes: selection of the biotic environment for floral features (sympatric congeners and types of pollinators) and the degree of floral response to this selection (its integration and precision). The attractiveness of these features, on the other hand, ensures that the pollinators attracted to a particular species are affected by these characters. Specialization along this path could result in coevolutionary pollinator attraction or pollinator switches (Armbruster and Muchhala, 2009). From our *Fritillaria* study, the foundation for these switches might be the quantity and quality of the reward offered to flower visitors.

Based on our *Fritillaria* data, such shifts seem unlikely to generate reproductive isolation that would allow sufficient divergence of populations by pollinator selection. Therefore, it is probable that bird-pollinated species of this genus like, for example, *F. imperialis* or *F. recurva* and *F. gentneri* are, and will continue to be, an intermediate phase during which both ancestral and new rewards and advertisements are present, and both ancestral and new pollinators visit the same flower (Armbruster and Muchhala, 2009). There is, still, a considerable need for further studies of *Fritillaria* pollination system in natural habitats and the genetic basis of character shifts in relation to their pollination system.

AUTHOR CONTRIBUTIONS

KR and MZ conceived the study and wrote the draft version of the paper. KR, LH, and PK assembled field data. KR, AB, MC, and AG performed the nectar analysis. KR, MZ, JS, MS, and LH analyzed the data. LH and KR assembled photographic documentation. All authors contributed to the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01246/full#supplementary-material

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Using Nectar-Related Traits to Enhance Crop-Pollinator Interactions

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Floral nectar and other reward facilitate crop pollination, and in so doing, increase the amount and breadth of food available for humans. Though abundance and diversity of pollinators (particularly bees) have declined over the past several decades, a concomitant increase in reliance on pollinators presents a challenge to food production. Development of crop varieties with specific nectar or nectar-related traits to attract and retain pollinating insects is an appealing strategy to help address needs of agriculture and pollinators for several reasons. First, many crops have specific traits which have been identified to enhance crop–pollinator interactions. Also, an improved understanding of mechanisms that govern nectar-related traits suggest simplified phenotyping and breeding are possible. Finally, the use of nectar-related traits to enhance crop pollination should complement other measures promoting pollinators and will not limit options for crop production or require any changes by growers (other than planting varieties that are more attractive or rewarding to pollinators). In this article, we review the rationale for improving crop-pollinator interactions, the effects of specific plant traits on pollinator species, and use cultivated sunflowers as a case study. Recent research in sunflower has (i) associated variation in bee visitation with specific floral traits, (ii) quantified benefits of pollinators to hybrid yields, and (iii) used genetic resources in sunflower and other plants to find markers associated with key floral traits. Forthcoming work to increase pollinator rewards should enable sunflower to act as a model for using nectar-related traits to enhance crop–pollinator interactions.

Keywords: nectar, bees, ecosystem services, yield, sunflower, sucrose, breeding, pollination

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NEED TO IMPROVE CROP–POLLINATOR INTERACTIONS

Production of most world crops depends on bees or other animals to provide or enhance pollination (Klein et al., 2007), an ecosystem service strongly influenced by floral nectar and other rewards. One attempt to assess the value of insect pollination in United States agriculture estimated US$30 billion (Calderone, 2012), while economic valuation of pollination worldwide was valued at €153 billion (Gallai et al., 2009). Though recent estimates of the importance of pollinators in agriculture appear careful and detailed, Melathopoulos et al. (2015) note that pollinator dependence of any single crop is confounded by effects of variety (genotype), environment, and management practices. Nevertheless, without wild and managed bees, various fruit and nut crops would be unavailable and other crops would be less abundant or more costly.

Several distinct trends suggest changes are needed to better manage crop pollination. Honey bee, (*Apis mellifera* L.), the single most significant pollinator worldwide, has suffered substantial declines in colony health and survival in North America and Europe (vanEngelsdorp and Meixner, 2010). Similar negative trajectories have been seen for diversity or abundance of wild bees (Biesmeijer et al., 2006; Potts et al., 2010), which are more important than honey bees for many crops (Garibaldi et al., 2013; Rader et al., 2016). Coincident with pollinator declines, global need for pollinators appears to be increasing, creating a mismatch between pollinator supply and demand (Aizen et al., 2008; Breeze et al., 2014). Following declines in pollinators, price increases in pollinator-dependent crops have been observed (Lautenbach et al., 2012), a trend that likely reflects increased costs of pollination, as per-hive rental fees for honey bees increased more than four fold in just over a decade (Johnson, 2010).

Efforts to address the imbalance of supply and demand for crop pollination logically depend on understanding the problem. Apparent causes for honey bee declines are varied, including diseases and parasites, exposure to pesticides, inadequate nutrition, and increasingly intensive use by humans (United States Department of Agriculture [USDA], 2013; Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services [IPBES], 2016). Explanations for negative trends in wild bee abundance or diversity are similar to those for honey bees, but with an emphasis on loss or degradation of non-crop habitats (Klein et al., 2007; Potts et al., 2010). In the United States, efforts to mitigate pollinator declines include improvement in practices related to honey bee health, restoration or enhancement of millions of hectares of land, and restriction or re-evaluation of pesticides (Pollinator Health Task Force, 2015; United States Environmental Protection Agency [US EPA], 2017). England's national strategy consists of largely voluntary and subsidized measures to support pollinators, including planting wildflowers on farmland and limiting pesticide use through promotion of integrated pest management (Department for Environment Food and Rural Affairs [DEFRA], 2014). One common implication of plans to conserve pollinators and pollination services is the recognition that many different types of measures are needed (Isaacs et al., 2017); with that in mind, crop breeding or selection of varieties that better attract and reward species that pollinate crops appears to be a neglected strategy (but see Palmer et al., 2009; Bailes et al., 2015) that could improve both crop yields and nutritional resources for pollinators.

EFFECTS OF NECTAR AND NECTAR-RELATED TRAITS ON CROP POLLINATORS

Nectar is the primary reward for pollinator visitation to wild and cultivated plants, and calories from nectar affect bee growth and development (Burkle and Irwin, 2009). Consequently, variation in nectar has obvious potential to influence pollinator behavior. However, the process of determining which traits are important for crop–pollinator interactions is complicated for several reasons. First, correlations among floral traits are relatively common (Davis, 2000); one trait assessed as influencing behavior in a crop may not be the trait of importance to a pollinator (e.g., flower size versus volume of floral nectar). Second, the state of one trait can easily mask other traits. For example, when floral morphology limits access to nectar (Hawkins, 1969; Erickson, 1975b), nectar quantity or quality are irrelevant to affected pollinators. As a result, nectar and nectar-related traits generally should not be considered to operate independently, but as combinations of reward, cues and other traits which determine plant interactions with pollinators and other insects (Raguso, 2004). Lastly, it is worthwhile to note that the effects of plant traits vary among pollinators; differences in life-histories (social versus solitary) or the identity of a single key pollinator species may determine the effect of nectar and nectar-related crop traits (Tepedino and Parker, 1982).

With the caveats regarding the complexity of crop–pollinator relationships in mind, examples of specific nectar-related traits associated with pollinator activity are noted under subheadings below. The types of traits discussed are well-established as influencing pollinator behavior in non-crop species, and (interspecific) variation in nectar-related traits of non-crop species is the basis for successful habitat manipulations to increase presence or activity of crop pollinators (Campbell et al., 2012; Feltham et al., 2015). Because our emphasis is on cultivated plants, many seminal publications on plant-pollinator interactions are not included. Also, the references are not an exhaustive list, but emphasize crops which show at least a modest increase in production through pollinator activity (see Klein et al., 2007) and studies that link intraspecific variation in nectar-related traits to a pollinator response.

Nectar Quantity and Quality

Intraspecific variation in the calories available to pollinators from nectar-feeding often helps explain pollinator preferences within a crop, and may arise from differences in nectar volume per flower, concentration of nectar sugars, density of flowers or the duration of flowering. Many fruit and vegetable crops with strong dependence on pollinators, including blueberry (Jabłoński et al., 1984), watermelon (Wolf et al., 1999), raspberries and blackberries (Schmidt et al., 2015), and zucchini (Roldán-Serrano and Guerra-Sanz, 2005) show positive associations between bee visits and nectar volume or total sugar per flower (nectar volume × concentration). Pollination benefits to *Citrus* species and cultivars vary, but nectar volume is correlated with honey bee visitation (and also flower size; Albrigo et al., 2012). Peppers and onions are both considered unattractive to bees and receive little direct benefit from pollinator visitation; however, bees are needed to produce hybrid seed, and increased honey bee visits are associated with increased nectar sugar or volume (Rabinowitch et al., 1993; Silva and Dean, 2000).

Unlike the aforementioned specialty crops, the enormous scale on which soybean is grown provides a significant opportunity to improve crop-pollinator interactions; though this legume is considered self-pollinated, some soybean varieties benefit from pollinator visitation (Erickson, 1975a) and show substantial variation in nectar volume (Erickson, 1975b;

Severson and Erickson, 1984). Soybean nectar and bee visits appear positively correlated (Erickson, 1975b; Robacker et al., 1983), but Palmer et al. (2009) suggest more work is needed to directly associate soybean floral traits with pollinator behavior. A similar situation exists for oilseed rape, which varies in nectar volume (Pierre et al., 1999; Bertazzini and Forlani, 2016), an attribute that increases the duration of bumblebee visits to flowers (Creswell, 1999).

Observations of bees foraging on sugar solutions (Waller, 1972; Mommaerts et al., 2013) and nectars from many plant taxa (Baker and Baker, 1983) suggest the ratio of common nectar sugars (sucrose, fructose, and glucose) may influence pollinator choice. Because sucrose is a disaccharide made of glucose and fructose, nectar sugar composition is often shown as a ratio of sucrose to fructose and glucose or as percent sucrose. Sucrose-richness of nectar in crops has only been implicated in pollinator choice for a few crops, including zucchini (Roldán-Serrano and Guerra-Sanz, 2005) and sunflower (Pham-Delègue et al., 1994). However, other crops including oilseed rape (Kevan et al., 1991) and peppers (Roldán-Serrano and Guerra-Sanz, 2004) have been shown to provide nectars that vary from no sucrose to sucrose-rich.

In addition to sugars, nectar contains a wide variety of other components at lower concentrations, including inorganic ions, amino acids, lipids, and secondary plant compounds (see Roy et al., 2017), many of which are attractant or repellent to pollinators. Few studies are available that examine intraspecific variation in these components, and even fewer which link the variation in non-sugar components of nectar to crop pollination. One interesting exception is caffeine; at levels found in coffee and citrus nectars, caffeine improves honey bee memory of a reward (nectar) and its associated cue (odor), suggesting caffeine encourages bees to make repeat visits to flowers of both plant genera (Wright et al., 2013). The amino acid proline, a floral nectar component, seems to increase honey bee preference at concentrations of 2–6 mM (Carter et al., 2006). Though oilseed rape shows significant variation in proline concentration (Bertazzini and Forlani, 2016), its levels may be below the 2 mM threshold to affect pollinator preference. On the other hand, accessions from a soybean wild relative suggest *Glycine* spp. may have proline levels high enough to influence bee foraging (Carter et al., 2006).

In addition to floral nectar, many cultivated plants have extrafloral nectaries. In general, extrafloral nectar is an inducible, indirect defense against herbivores that functions by attracting predators and parasitoids to damaged plants (Heil, 2015). Though extrafloral nectar has little apparent application for enhancing crop–pollinator interactions, it shares much of the quantitative and qualitative diversity found in floral nectar (González-Teuber and Heil, 2009). Because extrafloral nectaries benefit plants by reducing herbivory, Heil (2015) and Stenberg et al. (2015) suggest this indirect defense should be used in breeding crops.

Other Nectar-Related Traits

Floral scent and appearance also influence pollinator choice. Though there are innate pollinator preferences (Reverté et al., 2016), it is clear that bees use visual and olfactory information as indicators of floral reward, often learning to associate cues and reward. In wild *Brassica rapa*, the amount of the floral volatile phenylacetaldehyde was correlated with floral reward (sugar and pollen per flower), and bumble bees learned a positive response to the volatile after foraging on plants (Knauer and Schiestl, 2015). Preference of a wild bee for strawberry varieties was associated with higher levels of floral volatiles, but correlation of volatiles with reward was not tested (Klatt et al., 2013). Appearance of flowers is important for pollination of apple cultivars; when white-flowered apples were planted with several crabapples as pollen donors, honey bees showed a strong preference for white crabapples (Mayer et al., 1989), possibly due to flower constancy (a pollinator habit of repeatedly visiting one type of flower; Waser, 1986). Honey bees appear to evaluate alfalfa at a distance, as floral display size of individual plants positively influenced honey bee visitation (Bauer et al., 2017). Wild *Brassica rapa* and oilseed rape vary for the presence of nectar guides, an ultraviolet floral pattern visible to bees, which increase pollinator visits to plants (Brock et al., 2016). However, in one comparison, a mutation that causes complete loss of petals in oilseed rape did not appear to reduce honey bee visitation (Pierre et al., 1996).

Aside from providing visual cues, aspects of floral morphology can be important in limiting access to floral reward. The size of opening to access nectar ("throat diameter") was positively associated with honey bee visitation to highbush blueberry (Courcelles et al., 2013). Floral morphology in some soybean varieties strongly discourages pollinators by production of closed (cleistogamous) flowers; however, because flower type can be controlled by both genotype and environment, bee visitation to some varieties may occur in periodic pulses that coincide with production of open (chasmogamous) flowers (Erickson, 1975b).

Pollen is also a significant floral reward that shows intraspecific variation. The clearest instances where pollen appears to influence pollinator behavior are in male-sterile lines, which may receive more or less bee visits, depending on bee species or nutritional status (Tepedino and Parker, 1982; Soto et al., 2013). Few data are available to generalize how moderate quantitative differences in pollen (e.g., 25–35%; Vear et al., 1990) affect crop–pollination. A succinct summary of nectar-related crop traits and their effects on bees is shown in **Table 1**.

IMPROVING SUNFLOWER CROP YIELDS AND RESOURCES FOR BEES

Sunflowers are attractive to both managed and wild pollinators (United States Department of Agriculture [USDA], 2015), but because of selection for self-fertility, are sometimes considered to have a low-to-moderate dependence on bees (Delaplane and Mayer, 2000; Klein et al., 2007). However, for production of hybrid seed, where pollen must be moved between male-fertile and male-sterile lines, bees are critically important (Greenleaf and Kremen, 2006), and otherwise desirable inbred lines are sometimes discarded because of their

TABLE 1 | Nectar-related traits and pollinator responses for selected crops and crop wild relatives.

Species (common name)	Plant trait	Response	Reference
Allium cepa (onion)	Nectar volume	+ honey bee visits	Silva and Dean, 2000
Brassica napus (oilseed rape)	Nectar volume*	+ bumble bee visits	Creswell, 1999
Brassica napus (oilseed rape)	Absence of petals	= /+ honey bee visits	Pierre et al., 1996
Brassica rapa (field mustard)	Ultraviolet patterning	+ pollinator visits	Brock et al., 2016
Brassica rapa (field mustard)	Floral volatiles	+ bumble bee visits	Knauer and Schiestl, 2015
Capsicum annuum (pepper)	Nectar volume × concentration	+ honey bee visits	Rabinowitch et al., 1993
Citrus spp. (citrus)	Nectar volume, flower size	+ honey bee visits	Albrigo et al., 2012
Cucurbita pepo (zucchini)	Nectar volume, sugar ratios	+ bumble bee visits	Roldán-Serrano and Guerra-Sanz, 2005
Citrullus lanatus (watermelon)	Nectar concentration	+ honey bee visits	Wolf et al., 1999
Fragaria x *ananassa* (strawberry)	Floral volatiles	+ solitary bee visits	Klatt et al., 2013
Glycine max (soybean)	Flower access (cleistogamy)	− honey bee visits	Erickson, 1975b
Helianthus annuus (sunflower)	Nectar volume × concentration	+ social bee visits	Tepedino and Parker, 1982
Helianthus annuus (sunflower)	Nectar volume, flower size (depth)	+/− pollinator visits	Mallinger and Prasifka, 2017a
Helianthus annuus (sunflower)	Flower size (depth)	− wild bee visits	Portlas et al., 2018
Malus spp. (apple and crabapple)	Flower color	+ honey bees visits	Mayer et al., 1989
Medicago sativa (alfalfa)	Size of floral display	+ honey bee visits	Bauer et al., 2017
Rubus spp. (caneberries)	Nectar volume	+ social bee visits	Schmidt et al., 2015
Vaccinium corymbosum (blueberry)	Nectar volume × concentration	+ honey bee visits	Jabłoński et al., 1984
Vaccinium corymbosum (blueberry)	Flower size (diameter)	+ honey bee visits	Courcelles et al., 2013

**Surrogate nectar solution dispensed in a range of volumes after removal of naturally secreted nectar. Natural variation in nectar volume for B. napus shown by Pierre et al. (1999) and Bertazzini and Forlani (2016).*

failure to attract pollinators. Further, although commercial sunflower hybrids may be capable of self-pollination, yields are generally improved by bees (DeGrandi-Hoffman and Chambers, 2006). Traits associated with pollinator attraction and the pollinator-dependence of sunflower hybrids have been previously investigated (Tepedino and Parker, 1982; Sammataro et al., 1983; Pham-Delègue et al., 1994; Dag et al., 2002), but these studies often included few plant genotypes, were published outside of peer-reviewed literature, or used open-pollinated varieties developed without hybrid breeding. As a result, a series of studies has been undertaken by USDA-ARS researchers and collaborators to (i) associate variation in pollinator visitation with specific floral traits, (ii) assess benefits of pollinators to yields of modern sunflower hybrids, and (iii) use genetic resources in sunflower and other plants to facilitate improved sunflower-pollinator interactions. A summary of recently published and new data related to these objectives is provided below.

Field trials in 2014–2015 (Mallinger and Prasifka, 2017a) were designed to associate wild and managed bee visitation to floral traits of inbred lines. For pairs ($n = 10$) of sunflower isolines with or without cytoplasmic male sterility (cms), honey bees favored the pollen-free cms lines while wild bees preferred the male-fertile equivalents. After accounting for the effect of pollen, nectar sugar (volume × concentration) was positively associated with visits by both honey bees and wild bees. Additionally, inbred lines with shorter corollas (=easier access to nectar) were found to receive more pollinator visits. Subsequent work in 2016–2017 (Portlas et al., 2018) focused on the effect of floret size because deeper corollas prevent nectar sampling by short-tongued bees, and because phenotyping floret size should be more rapid and precise than assessing nectar volume. Evaluation of 100 female lines showed total floret length ranged from 6.8 to 9.9 mm. When a subset of these lines was grown again and bee visits counted daily, most of the variation in wild bee counts was explained by floret size. Data from Portlas et al. (2018) suggest that for lines with the longest florets, a reduction in floret size of only 1.0 mm should double bee visitation; further reductions in floret size beyond 1.0 mm provide even greater benefits, likely because proboscis ("tongue") lengths vary both within (Waddington and Herbst, 1987) and between bee species (Cariveau et al., 2016).

Over the same period, we evaluated pollinator contributions to sunflower yields. Because pollinator benefits to yields of oilseed hybrids were assessed somewhat recently (DeGrandi-Hoffman and Chambers, 2006), we focused on confection sunflowers (i.e., non-oil hybrids used as a snack food or as a food ingredient). Over 2 years in North Dakota, 15 commercial hybrids were grown with or without pollinators excluded (via fine mesh bags). Though some hybrids received no benefit from pollinators, open-pollination by insects increased yields by 26% when averaged across all hybrids, and five of the hybrids showed increases of 39–108% (Mallinger and Prasifka, 2017b). In part, variation in benefits from pollinators was explained by how attractive each hybrid was to bees, though nectar-related traits were not directly assessed for these hybrids. After repeating this work in additional states (South Dakota and Nebraska), early results indicate the effect of pollinators on yields may be greatly influenced by growing conditions. In 2016, yields from 10 tested hybrids saw a <20% increase from open pollination in North Dakota, a benefit of ≈30% in South Dakota and >100% increase over pollinator exclusion in Nebraska (Mallinger, unpublished data). Data from 2017 showed less variation across environments (pollinator benefit of 30–35%), but cumulative results indicate

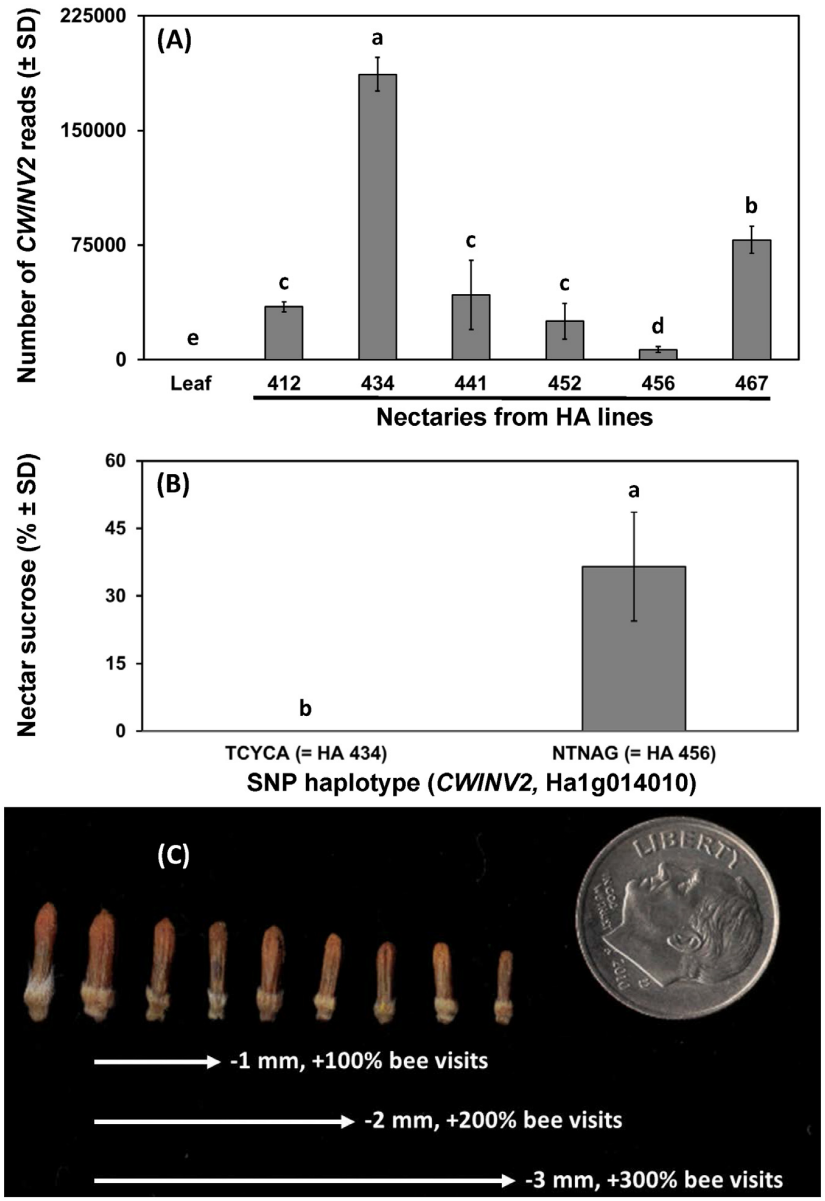

FIGURE 1 | Relationships between nectar-related traits in sunflower and genetic markers or pollinator behavior. Panels indicate **(A)** expression of cell wall invertase (*CWINV2*) from control tissue (leaf) and nectaries of *Helianthus annuus* maintainer (HA) lines previously phenotyped for sucrose content, **(B)** sucrose content (% by mass of sucrose + fructose + glucose) of nectars associated with two SNP haplotypes at *CWINV2* (n = 10 inbred lines per group), and **(C)** illustration of the range of floret sizes in cultivated sunflowers and the effect of decreasing floret size (from start to end of arrows) on visitation by wild bees as observed by Portlas et al. (2018). Significant differences between pairs in **(A,B)** indicated by differing lowercase letters.

that bees provide a substantial benefit to confection sunflower yields, and that even hybrids that effectively self-pollinate in one location or year may need bees to achieve consistent, high yields.

Given the importance of floral traits to bee visitation in sunflowers and the crop's reliance on bees, we attempted to leverage information on nectar-related traits in other plants and sunflower genetic resources to find and validate genetic markers that would enable marked-assisted breeding for improved sunflower-pollinator interactions. As a first step, we searched for sunflower homologues of *Arabidopsis thaliana* genes with known nectar-related functions (refer Table 2 from Roy et al., 2017), then examined whether variation in sunflower single nucleotide polymorhpisms (SNP) matched data on nectar volume or sugar composition from Mallinger and Prasifka (2017a). Observed phenotypic variation in inbred lines matched SNP markers from promoter or gene regions in just one case (cell wall invertase, *HaCWINV2*). When six inbred lines that varied for nectar volume and composition were grown (n = 4 replicates) and nectary gene expression quantified using

RNA-seq, results supported the hypothesis that *HaCWINV2* governs sucrose content in sunflower nectar, as the highest sucrose line (HA 456) showed the fewest reads (**Figure 1A**). To validate the gene-trait association in sunflowers, inbred lines with unknown nectar types but SNP haplotypes matching high or low sucrose lines (n = 10, each group) were grown and nectar sugars determined by high-performance anion exchange chromatography, which clearly supported the role of *CWINV2* in determining sugar composition in sunflower nectar (**Figure 1B**). While sucrose may influence bee foraging in sunflowers (Waller, 1972; Pham-Delègue et al., 1994; Mommaerts et al., 2013), finding markers for another nectar-related trait, floret size, is a priority because small changes in floret size have dramatic effects on nectar access (and sunflower visitation) by wild bees (**Figure 1C**; from data of Portlas et al., 2018). Previous identification of genes that govern flower size in other plants (Krizek and Anderson, 2013) suggests that this is achievable, and analysis of a broader panel of sunflower lines has identified several quantitative trait loci (QTL) for this trait (Hulke, unpublished data). Following identification of QTL that govern nectar quality and accessibility, the next challenges are to phenotype large populations for nectar and pollen quantity and develop markers which would expedite breeding sunflowers with enhanced pollinator reward.

FUTURE RESEARCH NEEDS

Research in sunflowers and other crops demonstrates that enhancement of crop-pollinator interactions by selection on nectar-related traits is both worthwhile and feasible. In addition to demonstrating potential benefits, trade-offs and costs also should be considered. For example, because adults and larvae of many insects feed on nectar and pollen (Wäckers et al., 2007), changes intended to benefit pollinators could also impact pest management (and vice-versa; Lucas-Barbosa, 2016). Also, targeted changes to nectar-related traits could have energetic costs that limit yields, though adaptations like nectar resorption can mitigate potential costs (Nepi and Stpiczyńska, 2007). However, given the potential benefits to crops and pollinators, trade-offs or costs should not discourage development of varieties and hybrids with improved nectar or nectar-related traits, but be addressed on a case-by-case basis.

AUTHOR CONTRIBUTIONS

JP, CC, KF, BH, and RM conducted the studies on sunflower nectar-related traits and designed the pollination. JP, ZP, MH, RM, CC, and TP collected and analyzed the data. JP, BH, RM, and ZP planned and wrote the manuscript. All authors have read and approved the manuscript.

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Sweet Scents: Nectar Specialist Yeasts Enhance Nectar Attraction of a Generalist Aphid Parasitoid Without Affecting Survival

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Floral nectar is commonly inhabited by microorganisms, mostly yeasts and bacteria, which can have a strong impact on nectar chemistry and scent. Yet, little is known about the effects of nectar microbes on the behavior and survival of insects belonging to the third trophic level such as parasitoids. Here, we used five nectar-inhabiting yeast species to test the hypothesis that yeast species that almost solely occur in nectar, and therefore substantially rely on floral visitors for dispersal, produce volatile compounds that enhance insect attraction without compromising insect life history parameters, such as survival. Experiments were performed using two nectar specialist yeasts (*Metschnikowia gruessii* and *M. reukaufii*) and three generalist species (*Aureobasidium pullulans*, *Hanseniaspora uvarum*, and *Sporobolomyces roseus*). *Saccharomyces cerevisiae* was included as a reference yeast. We compared olfactory responses of the generalist aphid parasitoid *Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) when exposed to these microorganisms inoculated in synthetic nectar. Nectar-inhabiting yeasts had a significant impact on nectar chemistry and produced distinct volatile blends, some of which were attractive, while others were neutral or repellent. Among the different yeast species tested, the nectar specialists *M. gruessii* and *M. reukaufii* were the only species that produced a highly attractive nectar to parasitoid females, which simultaneously had no adverse effects on longevity and survival of adults. By contrast, parasitoids that fed on nectars fermented with the reference strain, *A. pullulans, H. uvarum or S. roseus* showed shortest longevity and lowest survival. Additionally, nectars fermented by *A. pullulans* or *S. roseus* were consumed significantly less, suggesting a lack of important nutrients or undesirable changes in the nectar chemical profiles. Altogether our results indicate that nectar-inhabiting yeasts play an important, but so far largely overlooked, role in plant-insect interactions by modulating the chemical composition of nectar, and may

have important ecological consequences for plant pollination and biological control of herbivorous insects.

Keywords: floral nectar, *Metschnikowia*, nectar chemistry, microbial volatile (MVOC), *Aphidius ervi*, behavioral response, nectar intake, survival

INTRODUCTION

As a source of sugars, floral nectar is commonly colonized by nectarivorous microbes, most often yeasts and bacteria that may rapidly reach high densities within floral nectar (Herrera et al., 2009; Lievens et al., 2015; Pozo et al., 2015b). Although their precise ecological role is not yet entirely clear (Herrera, 2017), nectar-inhabiting microorganisms have a strong effect on nectar chemistry by altering the concentration and composition of sugars and amino acids (Herrera et al., 2008; de Vega et al., 2009; Canto and Herrera, 2012; Peay et al., 2012; Vannette et al., 2013; Lenaerts et al., 2017), and influencing acidity (Vannette et al., 2013; Good et al., 2014; Lenaerts et al., 2017). These changes may, in turn, affect the nectar's overall nutritional value and appeal to flower-visiting insects (Schaeffer et al., 2014, 2017; Lenaerts et al., 2017). The metabolic activity of nectar-inhabiting microbes has also been shown to affect other floral traits (Vannette and Fukami, 2016, 2018). Herrera and Pozo (2010), for example, showed that experimental addition of *Metschnikowia* yeasts to the nectar of a winter-blooming plant species (*Helleborus foetidus*) significantly increased the nectar temperature. Warmer nectar could offer energetic advantages for insect thermoregulation, as well as being easier to drink owing to its lower viscosity (Nicolson et al., 2013). Recent evidence also points to nectar-inhabiting microorganisms contributing to nectar scents by the production of volatile compounds (Golonka et al., 2014; Rering et al., 2017). It is generally believed that these microbial volatiles can act as semiochemicals that signal a suitable food source (nectar) or habitat to nectar feeding insects (Wright and Schiestl, 2009; Davis et al., 2013), while the microbes benefit from the insects as vectors for dispersal to new environments (Christiaens et al., 2014). The plants may benefit from the presence of microorganisms through increased insect visitation rates or longer foraging time (Schaeffer et al., 2017), ultimately leading to enhanced plant fitness (Schaeffer and Irwin, 2014). However, effects on plant fitness seem to vary depending on the component of plant reproduction considered (Herrera et al., 2013).

For microbes that strongly rely on animal vectors, such as insects, production of insect-attractive volatiles may be an efficient strategy to rapidly disperse and colonize new habitats, while this would be less needed for generalist microbes that live in a wider variety of habitats and are less reliant on insect vectors (Dzialo et al., 2017). Indeed, it was recently shown that *Metschnikowia reukaufii*, a yeast species that is specialized to thrive in the harsh nectar environment (Lievens et al., 2015; Pozo et al., 2015a) and is largely dependent on flower-visiting insects for dispersal (Belisle et al., 2012; Vannette and Fukami, 2017), produces distinctive volatile compounds. It was also demonstrated that this yeast species is more attractive to honey bees (*Apis mellifera*) than generalist microorganisms (Rering et al., 2017). Nevertheless, as the authors only examined responses of one floral visitor, additional research is needed to generalize these results. Another important group of flower-visiting insects are hymenopteran parasitoids. Parasitic Hymenoptera represent a key factor in regulating natural insect populations, and form an important component in biocontrol programs of insect pests (Narendran, 2001). Like bees, in their adult stage, parasitoid wasps feed on carbohydrate-rich food such as floral nectar to cover their energetic and nutritional needs (Jervis et al., 1993). This makes them ideal candidates for further study of the role of nectar microbes in the foraging behavior of flower-visiting insects. Moreover, social Hymenoptera, such as honey bees and bumble bees, have the disadvantage that isolating them from their social interactions within the colony may negatively impact foraging behavior (Garibaldi et al., 2011), food consumption (Arnold, 1979), and survival (Sitbon, 1967). Furthermore, it can be hypothesized that microorganisms that substantially rely on insect vectors for dispersal should not impair life history parameters of their vectors, such as survival. In a recent study, it was found that different nectar bacteria may have a clear effect on the longevity of flower-visiting insects by altering nectar chemistry (Lenaerts et al., 2017), but it remains unclear whether effects can be related to the ecology of the microorganisms.

Here, we tested the hypothesis that yeast species that almost exclusively occur in nectar and therefore largely depend on floral visitors for dispersal produce volatile compounds that enhance insect attraction, and simultaneously yield a nectar chemistry that does not harm the survival of attracted insects. To this end, we used the nectar specialists *Metschnikowia gruessii* and *M. reukaufii* as model yeasts. In addition, we tested more generalist yeast species such as *Aureobasidium pullulans*, *Hanseniaspora uvarum* and *Sporobolomyces roseus* (Lievens et al., 2015; Pozo et al., 2015b). *Saccharomyces cerevisiae* (Y182), which is not found in nectar, but is known for its high aroma production and attraction of *Drosophila* flies (Christiaens et al., 2014), was used as a reference. All experiments were performed using the solitary hymenopteran parasitoid *Aphidius ervi* (Haliday), which is a generalist parasitoid of aphids. It feeds preferentially on nectar as a main source of sugars over honeydew (Vollhardt et al., 2010), and its efficiency in suppressing aphid populations is drastically increased by the provision of floral nectar (Araj et al., 2008, 2011). First, we investigated the effect of the different nectar-inhabiting yeasts (NIYs) on the volatile production and chemical composition of a synthetic nectar solution mimicking real nectar. Next, using a binary olfactory choice assay, the parasitoid response to the NIY-fermented nectars was assessed. Finally, using a capillary feeder (CAFE) assay, the intake of NIY-fermented nectars by parasitoid adults and the subsequent impact on their longevity and survival were studied.

MATERIALS AND METHODS

Study Organisms

Yeasts

Five yeast strains that were previously isolated from floral nectar of wild plants were used in this study (Table S1), including two nectar specialist species (*M. gruessii* and *M. reukaufii*) and three generalist species (*A. pullulans, H. uvarum*, and *S. roseus*) (Jacquemyn et al., 2013; Lenaerts et al., 2016b). *Metschnikowia reukaufii* and *M. gruessii* are common and abundant inhabitants of floral nectar that have specialized on the nectar environment (Herrera et al., 2009; Lievens et al., 2015; Pozo et al., 2016). Additionally, both species are strongly dependent on floral visitors for transmission among flowers, including pollinators and parasitoids (e.g., Belisle et al., 2012; Pozo et al., 2012; Herrera et al., 2014; Srinatha et al., 2015). By contrast, the other three yeast species have a broader habitat range and are less dependent on insect vectors for dispersal. More specifically, *A. pullulans* is a ubiquitous yeast-like fungus that can be found in different environments including soil, water, air, and in or on plants (Andrews et al., 1994). *Hanseniaspora uvarum* is an apiculate yeast species that is frequently found on mature fruits (Jolly et al., 2014), whereas *S. roseus* is mostly associated with the phyllosphere (Nakase, 2000). Additionally, as a reference we included a *S. cerevisiae* strain (Y182) that produces strong aroma (e.g., aroma-active esters) and has been shown to attract *Drosophila melanogaster* fruit flies (Christiaens et al., 2014). Yeast strains were stored at −80°C in yeast extract peptone dextrose broth (YPDB; Difco, Le Pont-de-Claix, France) containing 37.5% glycerol.

Insects

Experiments were performed with adults of *A. ervi* (Hymenoptera: Braconidae). *Aphidius ervi* is a solitary generalist endoparasitoid that attacks many aphid species, including numerous species of economic importance (van Lenteren, 2012). For all experiments, *A. ervi* mummies were supplied by Biobest (Ervi-system®, Westerlo, Belgium). Upon receiving, mummies were either kept at 4°C for a maximum of 48 h until usage or placed directly in a nylon insect cage (20 × 20 × 20 cm, BugDorm-41515, MegaView Science Co., Ltd., Taichung, Taiwan) and kept under controlled conditions (22°C, 70% relative humidity, 16:8 h light:dark photoperiod) until adult emergence. Prior to starting experiments, insects were subjected to a dark period of 8 h. All experiments were performed with feeding-inexperienced and water-starved adults that were <24 h old.

Inoculation and Fermentation of Synthetic Nectar

In order to prepare different yeast-fermented nectars, stock cultures were plated on yeast extract peptone dextrose agar (YPDA), followed by a re-streak on the same medium and subsequent incubation for 2 days at 25°C. Yeast strains were thereafter inoculated in a test tube containing 5 ml YPDB and incubated at 25°C on a rotary shaker at 150 rpm. After overnight incubation, cells were washed two times and suspended in sterile physiological water (0.9% NaCl) until an optical density (OD 600 nm) of 1 was reached. Afterwards, 1.5 ml of this suspension was used to inoculate a 250 ml Erlenmeyer flask containing 150 ml sterile synthetic nectar prepared by filter-sterilizing 15% w/v sucrose solution supplemented with 3.16 mM amino acids from digested casein (Vannette and Fukami, 2014; Lenaerts et al., 2017). Erlenmeyer flasks were sealed with fermentation water locks and incubated statically at 25°C for 7 days. The incubation period was determined by regularly monitoring yeast growth to obtain densities that were comparable with those observed in floral nectar (de Vega et al., 2009). Each fermentation was performed in duplicate, and a medium without yeast inoculation was included as a mock control (which was also confirmed to be free of yeasts and bacteria after the fermentation). Following the fermentations, yeast-fermented nectars were centrifuged at 6,000 rpm for 3 min and subsequently filtered (pore size 0.22 μm; Nalgene, Waltham, MA, USA) to obtain cell-free cultures. Cell-free nectar media were then stored in small aliquots in sterile dark glass vials (Fagron, Nazareth, Belgium) at −20°C until further use.

Impact of Yeasts on Scent Profiles

In order to investigate the effects of the different yeast strains on nectar scent, fermented nectars were subjected to a headspace gas chromatography (GC) analysis coupled with a flame ionization detector (HS-GC-FID; Shimadzu, Kyoto, Japan) as described previously (Christiaens et al., 2014). For each biological replicate of fermented nectars, the analysis was performed with two technical replicates. The GC was calibrated for 15 important yeast specific volatiles, including esters (ethyl acetate, isobutyl acetate, propyl acetate, isoamyl acetate, phenyl ethyl acetate, ethyl propionate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate), higher alcohols (isoamyl alcohol, isobutanol, butanol, phenyl ethanol) and acetaldehyde as described in Gallone et al. (2016). The GC was fitted with the DB-WAX column (30 m length × 0.32 mm inner diameter × 0.5 μm film thickness, Agilent Technologies, Santa Clara, CA, USA). Samples of 5 ml fermented nectar were collected in 15 ml glass tubes containing 1.75 g of sodium chloride each. These tubes were immediately closed and cooled to minimize evaporation of volatile compounds. The injector port of the GC instrument was held at 250°C *via* a headspace auto sampler (PAL system; CTC Analytics, Zwingen, Switzerland). N_2 was used as the carrier gas. The GC oven temperature was programmed at 50°C for 5 min, after which it was increased to 80°C at 5°C min^{-1}. Next, the temperature was increased to 200°C at 4°C min^{-1} and held at 200°C for 3 min followed by a final ramp of 4°C min^{-1} till 230°C. Results were analyzed with the GCSolution software version 2.4 (Shimadzu, Kyoto, Japan).

Impact of Yeasts on Nectar Chemistry

To investigate the effects of the different yeast strains on nectar chemistry, prepared synthetic nectar media were subjected to chemical analyses. In particular, concentrations of sugars and amino acids were determined with a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Thermo Fisher Scientific Dionex, Sunnyvale, CA,

USA) as described by Lenaerts et al. (2016a). Furthermore, the pH was determined with a pH electrode (WTW Inolab, Weilheim, Germany) and the Gallery™ Plus Beermaster (ThermoFisher, Vantaa, Finland) was used to quantify acetic acid, D-Lactic acid and sulfur dioxide levels. Concentrations were calculated from a calibration curve generated using standards according to the manufacturer's instructions. Analyses were performed with two technical replicates per nectar medium (i.e., per biological replicate).

Impact of Yeast-Fermented Nectars on Insect Behavioral Response

In order to assess the effect of yeast-fermented nectar on *A. ervi* foraging behavior, a behavioral bioassay using a glass Y-tube olfactometer was performed. The olfactometer consisted of a 20-cm-long stem tube with 1.5 cm inner diameter and two 12-cm-long lateral arms with a 60° angle at the Y-junction. Charcoal filtered humidified and purified air was provided at 400 ml min^{-1} (Brooks Instrument flow meter, Hatfield, USA) to both branches of the Y-tube *via* two odor chambers using a vacuum pump (Tetratec APS 150, Mella, Germany). All connections in the olfactometer were made using Teflon tubing (Figure S1). To test a given yeast strain, a filter paper (Macherey-Nagel, Düren, Germany) was loaded with 150 µl cell-free fermented nectar and placed into one of the two odor chambers of the olfactometer, whereas in the second chamber another filter paper was placed on which 150 µl non-inoculated medium was added. The bioassay was carried out by releasing 20 groups of five adult females ($n = 100$), in one experimental day, at the base of the olfactometer and evaluating their response 10 min after their release. Wasps that passed a set line at the end of the olfactometer arms (1 cm from the joint) and remained there at the time of evaluation were considered to have chosen for the odor source connected to that olfactometer arm. Parasitoids that did not make a choice within 10 min after release were considered as non-responding individuals, and were excluded from the statistical analysis. In order to avoid light-bias, the experiment was conducted in a 60 × 40 × 25-cm white chamber that was illuminated with four warm white led 5.5 W lamps (EGLO E27, light intensity 1880 lumens). Further, to avoid positional bias, the odor chambers were rotated after 10 releases with a new set of Teflon tubes. The glass Y-tube was renewed by a cleaned tube (see below) after every five runs, to eliminate choices which may be based on potential insect traces. Filter papers were replaced with fresh filter papers with 150 µl of the tested medium every two runs to maintain a high level of odor release. At the end of the experiment, all olfactometer parts (glass and Teflon tubes) were thoroughly cleaned with tap water and then distilled water, acetone (Forever, Courcelles, Belgium; purity > 99%) and finally pentane (Sigma-Aldrich, Steinheim, Germany; purity 98%). After solvents had evaporated, the glass parts were placed overnight in an oven at 150°C. All bioassays were conducted at 20 ± 1°C, 60 ± 5% RH and performed between 09:00 and 16:00 h.

Impact of Yeast-Fermented Nectars on Nectar Intake, Longevity, and Survival

The capillary feeder (CAFE) assay that was previously developed by Lenaerts et al. (2016a) was used to evaluate the effect of yeast inoculation on nectar intake and parasitoid longevity and survival. In brief, a cylindrical plastic container (height: 12.5 cm; diameter: 10 cm) was provided with four calibrated glass micropipettes (5.0 µl, Blaubrand IntraMARK, Wertheim, Germany) that were filled with 4.0 µl of the cell-free nectar solution fermented by one of the tested yeast strains (no-choice; all four capillaries contained the same nectar solution) and covered with 1.0 µl inert mineral oil to minimize evaporation. Additionally, a treatment with nectar that had not been inoculated with yeast was included as a control. Filled capillaries were inserted through the lid *via* truncated 200-µl yellow pipette tips to orientate the wasps to the microcapillaries (Battaglia et al., 2000). Further, to allow ventilation the lid of the container was pierced and covered with a fine mesh (2.5 × 2.5 cm; mesh size 0.27 × 0.88 mm). To provide parasitoids with sufficient water and humidity, a filter paper saturated with 500 µl of sterile water was put at the container's bottom, which was supplemented daily with an additional 200 µl of sterile water. Experiments were performed using a group of 75 individuals (both males and females) that were divided equally over five containers per treatment (15 individuals per cage) in a controlled environment (Micro Clima-Series™, Economic Lux Chamber, Snijders LABS, Tilburg, The Netherlands). Experiments were conducted at 22°C, 70% RH and a 16:8-h light: dark photoperiod with a light intensity of 100 µmol/m^2 s during periods of light. For the first 9 h of the experiment, nectar intake was assessed every hour by measuring the nectar column in the microcapillaries using a digital caliper (Mitutoyo Digimatic, resolution 0.01 mm). For each CAFE feeder, consumption values for the four capillaries were summed and subsequently averaged over the five replicates ($n = 5$). To determine the exact starting volume, we also measured the level of nectar solution right before the start of the experiment. An additional identical feeding unit, but without parasitoids, was included for each treatment to establish losses through evaporation. These values were then subtracted from experimental readings to account for evaporative losses.

Further, the effect of the different yeast-fermented nectars on insect longevity (days from adult emergence until death) and survival (number or proportion of adults surviving under the testing conditions) was assessed using the same individuals as those used in the previous analysis ($n = 75$). More specifically, parasitoid longevity was recorded daily by counting and removing the dead individuals in each CAFE container, until the last individual had died. To avoid microbial contamination of the nectar solutions, capillaries were replaced daily.

Data Analysis and Visualization

All analyses of nectar volatiles and nectar chemistry were performed using two biological replicates which were analyzed each in duplicate (two technical replicates). Variation between both biological and technical replicates was low (Data Sheet 1), illustrating the robustness of our data, as has also been shown previously (Christiaens et al., 2014). For each biological replicate, we used the mean values of the two technical replicates to run the statistical analysis. First, changes in nectar chemistry (MVOCs, amino acids, sugars and acidity) by yeast fermentation

were visualized using a principal component analysis (PCA), incorporating each compound as a variable according to Rencher (2002). We used two types of output: a matrix of "scores," which provides the location of each sample on each PC, and a matrix of "loadings" which indicates the strength of correlation between individual compounds and each PC. Prior to analysis, data were normalized by sum, cube root transformed and mean-centered, and divided by the standard deviation of each variable before PCA, using the comprehensive online tool suite MetaboAnalyst 3.0 (Xia et al., 2015). Next, to get better insight into the changes in nectar profiles (i.e., MVOCs, amino acids, sugars and acidity) upon yeast inoculation, data were analyzed using one-way ANOVA for each individual compound. Data were first checked for normal distribution and homogeneity of variance by Shapiro–Wilk and by Levene's test, respectively. The obtained *P*-values were adjusted for multiple testing, by the Benjamini and Hochberg (BH) step-up procedure to control the false discovery rate (FDR) (Benjamini and Hochberg, 1995). As differences in nectar chemistry between biological replicates were small, insect experiments were performed using sampled nectar from one of both biological replicates.

To examine the effect of the different nectars on parasitoid foraging behavior, parasitoid response was analyzed under the null hypothesis that adult parasitoids show no preference for either olfactometer arm (i.e., 50:50 response). Data ($n = 100$) were checked first for normality using Shapiro–Wilk test, after which they were analyzed with a *t*-test. The data ($n = 5$) of total nectar consumption during the first 9 h were analyzed using one-way ANOVA and means were then compared using a Student-Newman-Keuls *post-hoc* test. Longevity data ($n = 75$) were analyzed using one-way ANOVA which was followed by a Student-Newman-Keuls *post-hoc* test to compare means. Kaplan-Meier survival analysis and log-rank tests with Bonferroni correction were used to compare the survival of *A. ervi* adults fed on the various yeast-fermented nectars.

All the univariate analyses were performed using the statistical package SigmaPlot 12.3 (SYSTAT Inc., Chicago, IL, USA).

Ethical Note

Experimental manipulation of parasitoids occurred according to the common and ethical requirements for animal welfare. All parasitoids were carefully handled during experiments and maintained in the laboratory under appropriate conditions.

RESULTS

Impact of Yeasts on Scent Profiles

Analysis of the MVOCs that were collected from the different yeast-fermented nectars revealed differences in the nectar volatile composition and quantity (**Table 1**). In total, 13 MVOCs were detected for the different NIYs. As anticipated, the total amount of MVOCs emitted by the reference strain (Y182) was significantly higher compared to the other yeasts ($H_6 = 12.62$, $P < 0.001$), particularly due to the high emission of acetaldehyde [$F_{(6, 13)} = 272.67$, $P = 0.0036$], 2-methyl propanol ($H_6 = 12.08$, $P = 0.0077$) and 3-methyl-1-butanol [$F_{(6, 13)} = 123.19$, $P = 0.0145$]. PCA of the MVOCs showed that the first two components accounted for 77.8% of the total variation in volatile data (**Figure 1A**). Overall, PCA revealed that, compared to the control nectar, largest differences were for *A. pullulans, M. gruessii*, and *M. reukaufii*-fermented nectars. By contrast, the volatile blends emitted by *H. uvarum* and *S. roseus* only marginally differed from the control nectar. Furthermore, a noticeable separation was found between nectar fermented with NIYs and the reference strain Y182 (**Figure 1A**). The greatest loadings of PC1, in descending order, were for isoamyl acetate (0.335), ethyl propionate (0.334), isobutyl acetate (0.334) and 2-methyl propanol (0.329), whereas the greatest loadings of PC2 were for ethyl butyrate (0.425), 3-methyl-1-butanol (0.334), methanethiol (0.313) and 2-phenyl ethanol (0.253).

Impact of Yeasts on Nectar Chemistry

Amino acids concentration and composition were significantly affected by inoculation of yeast strains (**Table 1**). In particular, Y182 and *M. gruessii* significantly reduced the total amino acids content [$F_{(6, 13)} = 10.73$, $P = 0.003$] by an average of 36.5 and 19.3%, respectively. In contrast, *S. roseus* was the only yeast that increased, albeit marginally, total amino acids content by an average of 11.7% compared to the control nectar, especially glutamic acid and alanine (**Table 1**). The multivariate analysis (PCA) of amino acids showed that the first two components accounted for 76.1% of the total variation in amino acids data (**Figure 1B**). Overall, PCA revealed a very clear separation among the amino acid profiles from Y182, *A. pullulans* and *S. roseus*-fermented nectar compared to the control and nectar fermented by the other tested yeasts (**Figure 1B**). The greatest loadings of PC1 were for alanine (0.297), phenylalanine (0.283) and valine (0.276) and, whereas the greatest loadings of PC2 were for methionine (0.543), iso-leucine (0.448) and leucine (0.422).

A similar trend was observed for sugars (**Table 1**). Both Y182 and *A. pullulans* significantly reduced sucrose ($H_6 = 11.943$, $P < 0.001$) concentrations by an average of 33.9 and 89.5%, respectively, compared to the other nectars including the control. Furthermore, all yeast strains significantly increased glucose and fructose concentrations, especially Y182 and *A. pullulans* (**Table 1**). PCA showed that the first two components accounted for 99.8% of the total variation in the sugars data. Again, the largest separation was seen for Y182 and *A. pullulans* where both fructose and glucose vectors were more associated with the samples of these yeast-fermented nectars (**Figure 1C**), whereas the sucrose vector was more associated with the control and other tested yeasts.

Furthermore, it was found that all tested yeast strains significantly decreased nectar pH [$F_{(6, 13)} = 10.74$, $P = 0.05$], particularly Y182 and *A. pullulans* which reduced the pH from 5.76 to 4.07 and 3.91, respectively (**Table 1**). Additionally, Y182 drastically increased the concentration of acetic acid [$F_{(6, 13)} = 28.92$, $P = 0.0125$], D-Lactic acid [$F_{(6, 13)} = 28.42$, $P = 0.0250$] and sulfur dioxide [$F_{(6, 13)} = 16.26$, $P = 0.0375$] compared to the other tested yeasts. The multivariate analysis (PCA) of these compounds showed that the first two components accounted for 89.7% of the total variation in organic acids and sulfur dioxide data (**Figure 1D**), and clearly demonstrated differences between

TABLE 1 | Chemical profiles of synthetic nectar inoculated with various nectar-inhabiting yeasts.

Category	Class	Compound	Unit	Nectar-inhabiting yeasts*							*P*-value#
				Control	Y182	A.p.	H.u.	M.g.	M.r.	S.r.	
Acidity			pH	5.76^{a}	4.07^{b}	3.91^{c}	4.94^{ab}	5.08^{a}	4.48^{b}	4.32^{b}	**0.05**
Acids		Acetic acid	mg/l	0.004^{b}	0.152^{a}	0.008^{b}	0.018^{b}	0.023^{b}	0.039^{b}	0.047^{b}	**0.0125**
		D-Lactic acid	mg/l	1.34^{b}	11.41^{a}	2.58^{b}	1.46^{b}	1.26^{b}	1.23^{b}	2.11^{b}	**0.0250**
Amino acids	Acidic and their amides	Aspartic acid	mmol/l	234.73^{b}	23.35^{d}	280.42^{a}	199.34^{c}	214.27^{b}	236.06^{b}	262.7^{a}	**0.0029**
		Asparagine	mmol/l	0.44^{c}	1.96^{a}	1.46^{b}	0.61^{c}	1.43^{b}	1.27^{b}	0.89^{bc}	**0.0088**
		Glutamic acid	mmol/l	374.82^{b}	15.14^{c}	345.41^{b}	345.45^{b}	310.65^{b}	310.34^{b}	452.59^{a}	**0.0059**
		Glutamine	mmol/l	0.26^{c}	13.77^{a}	7.12^{b}	4.15^{b}	1.89^{bc}	2.85^{bc}	4.86^{b}	**0.0118**
	Aliphatic	Alanine	mmol/l	97.08^{b}	153.11^{a}	133.63^{a}	116.6^{ab}	65.92^{c}	92.45^{b}	130.07^{a}	**0.0147**
		Arginine	mmol/l	36.94^{a}	0.34^{b}	17.31^{a}	26.07^{a}	35.14^{a}	36.19^{a}	18.9^{a}	**0.0206**
		Glycine	mmol/l	60.93^{a}	65.5^{a}	64.15^{a}	56.93^{a}	48.09^{b}	58.075^{a}	69.87^{a}	**0.0471**
		Iso-Leucine	mmol/l	60.11^{ab}	62.72^{ab}	64.21^{ab}	79.46^{a}	46.83^{b}	52.69^{b}	61.6^{ab}	**0.0441**
		Leucine	mmol/l	102.22	94.98	112.45	119.74	81.93	91.32	113.59	0.05
		Valine	mmol/l	105.57^{a}	149.02^{a}	126.28^{a}	149.36^{a}	90.17^{b}	103.67^{a}	116.19^{a}	**0.0353**
	Aromatic	Phenylalanine	mmol/l	53.62^{b}	61.01^{ab}	68.45^{a}	61.56^{ab}	40.71^{c}	45.69^{c}	63.23^{a}	**0.0294**
		Tyrosine	mmol/l	30.41^{a}	35.63^{a}	36.42^{a}	40.94^{a}	26.16^{b}	30.82^{a}	35.71^{a}	**0.0412**
	Basic	Histidine	mmol/l	24.07^{a}	10.95^{b}	22.31^{a}	31.04^{a}	22.79^{a}	22.53^{a}	25.9^{a}	**0.0324**
		Lysine	mmol/l	106.91^{a}	51.87^{b}	64.67^{b}	121.84^{a}	107.28^{a}	109.03^{a}	120.38^{a}	**0.0176**
	Containing OH group	Serine	mmol/l	118.35^{a}	125.82^{a}	95.16^{b}	75.99^{c}	65.41^{c}	85.42^{b}	110.58^{ab}	**0.0235**
		Threonine	mmol/l	75.87^{a}	75.03^{a}	91.12^{a}	80.85^{a}	43.07^{b}	54.49^{b}	78.71^{a}	**0.0265**
	Containing sulfur	Methionine	mmol/l	39.14^{ab}	26.56^{b}	27.67^{b}	48.81^{a}	26.46^{b}	27.86^{b}	34.34^{b}	**0.0382**
MVOCs	Alcohol	2-Methyl propanol	mg/l	ND^{c}	22.238^{a}	3.537^{b}	0.058^{c}	0.038^{c}	0.392^{c}	0.036^{c}	**0.0077**
		3-Methyl-1-butanol	mg/l	ND^{d}	5.806^{a}	1.172^{bc}	0.167^{d}	0.901^{c}	1.777^{b}	0.073^{d}	**0.0145**
		2-Phenyl ethanol	mg/l	ND	1.461	0.318	0.377	1.643	0.391	0.203	0.0462
	Aldehyde	Acetaldehyde	mg/l	0.014^{c}	21.987^{a}	4.419^{b}	0.262^{c}	0.069^{c}	0.467^{c}	0.029^{c}	**0.0038**
	Ester	Amyl acetate	mg/l	ND^{b}	0.006^{a}	ND^{b}	ND^{b}	0.006^{a}	ND^{b}	ND^{b}	**0.0308**
		Ethyl acetate	mg/l	ND^{c}	0.076^{ab}	0.001^{c}	0.096^{a}	ND^{c}	0.002^{c}	0.035^{b}	**0.0346**
		Isoamyl acetate	mg/l	ND^{b}	0.084^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	**0.0296**
		Propyl acetate	mg/l	ND^{c}	0.035^{a}	ND^{c}	0.009^{c}	ND^{c}	ND^{c}	0.018^{b}	**0.0115**
		Isobutyl acetate	mg/l	ND^{b}	0.022^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	**0.0192**
		Ethyl butyrate	mg/l	ND^{c}	0.048^{b}	0.118^{a}	ND^{c}	ND^{c}	0.011^{c}	ND^{c}	**0.0231**
		Ethyl propionate	mg/l	ND	0.038	ND	ND	ND	ND	ND	0.0324
	Containing sulphur	Dimethyl disulfide	μg/l	ND	1.037	ND	ND	ND	ND	0.019	0.0423
		Methanethiol	μg/l	ND	0.295	0.242	0.227	0.211	0.561	0.209	0.05
Sugars	Monosaccharide	Glucose	mmol/l	4.93^{e}	208.68^{b}	313.75^{a}	25.87^{d}	17.86^{d}	19.01^{d}	45.58^{c}	**0.0167**
		Fructose	mmol/l	3.95^{d}	224.57^{a}	68.03^{b}	26.68^{c}	18.76^{c}	21.17^{c}	45.88^{b}	**0.05**
	Disaccharide	Sucrose	mmol/l	434.01^{b}	286.78^{d}	45.81^{e}	480.04^{a}	403.11^{c}	421.97^{b}	419.57^{b}	**0.0333**
Sulphur		Sulphur dioxide	mg/l	0.05^{c}	0.29^{a}	0.16^{b}	0.07^{c}	0.16^{b}	0.12^{bc}	0.23^{ab}	**0.0375**

Presented values are means of two biological replicates, on which two measurements were performed (two technical replicates). Different letters within rows indicate statistically significant differences ($P \leq 0.05$); when no letters are present there were no significant differences between treatments.

**Nectar-inhibiting yeasts were: Control, non-inoculated, yeast-free nectar; Y182, fermented nectar with Saccharomyces cerevisiae; A.p., fermented nectar with Aureobasidium pullulans; H.u., fermented nectar with Hanseniaspora uvarum; M.g., fermented nectar with Metschnikowia gruessii; M.r., fermented nectar with M. reukauffii; S.r., fermented nectar with Sporobolomyces roseus. MVOCs were identified according to retention times on DB-WAX column in comparison with synthetic standards. #Adjusted P-values as calculated after correcting for multiple comparisons by the Benjamini and Hochberg method; bold fonts indicate statistical significance.*

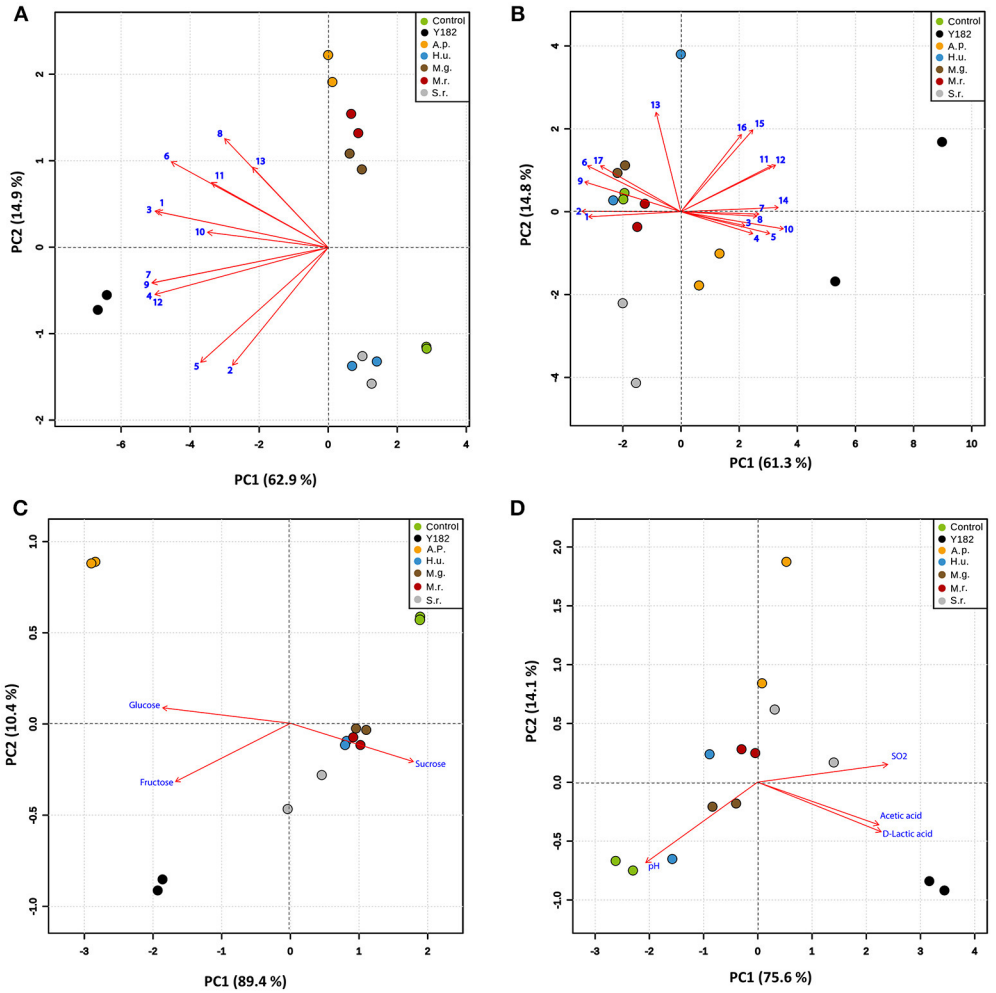

FIGURE 1 | Principal component analysis (PCA) of the volatile and chemical profiles of the different nectars, including: Control, non-inoculated, yeast-free nectar; Y182, nectar fermented with the reference strain *Saccharomyces cerevisiae* Y182; A.p., *Aureobasidium pullulans*-fermented nectar; H.u., *Hanseniaspora uvarum*-fermented nectar; M.g., *Metschnikowia gruessii*-fermented nectar; M.r., *Metschnikowia reukaufii*-fermented nectar; S.r., *Sporobolomyces roseus*-fermented nectar. All analyses were performed on cell-free nectar solutions (two biological replicates, each with two technical replicates; mean values for each biological replicate are used in the analysis). Score plots visualize the location of each analyzed sample on each PC with the percentage of explained variation in parentheses, whereas vectors (in red) visualize the loadings for each variable. **(A)** PCA showing variation in microbial volatile composition across the different treatments. Vector numbers refer to the different volatile compounds: (1) Acetaldehyde, (2) Ethyl acetate, (3) 2-Methyl propanol, (4) Ethyl propionate, (5) Propyl acetate, (6) 3-Methyl-1-butanol, (7) Isobutyl acetate, (8) Ethyl butyrate, (9) Isoamyl acetate, (10) Amyl acetate, (11) 2-Phenyl ethanol, (12) Dimethyl disulfide, and (13) Methanethiol. **(B)** PCA showing variation in the amino acids composition across different treatments. Vector numbers refer to the different amino acids: (1) Aspartic acid, (2) Glutamic acid, (3) Asparagine, (4) Serine, (5) Glutamine, (6) Histidine, (7) Glycine, (8) Threonine, (9) Arginine, (10) Alanine, (11) Tyrosine, (12) Valine, (13) Methionine, (14) Phenylalanine, (15) Iso-Leucine, (16) Leucine and (17) Lysine. **(C)** PCA showing variation in the sugar composition across the different nectars investigated. **(D)** PCA showing variation in acidity (pH), and the acids and sulfur dioxide composition in the different nectars. The percentage of variation of the data explained by PC1 and PC2 is shown in parentheses (**A** volatiles: 62.9 & 14.9%; **B** Amino acids: 61.3 % & 41.8 %; **C** Sugars: 89.4 & 10.4 %; **D** Acids: 75.9 & 14.1 %, respectively).

the control nectar and nectar fermented by yeasts, especially for Y182 and *A. pullulans*.

Impact of Yeast-Fermented Nectars on Insect Behavioral Response

Overall, yeast-fermented nectar elicited strong attraction of *A. ervi* parasitoid females compared to the control in a binary choice assay [$t_{(38)} = 2.240$, $P = 0.026$; **Figure 2**]. Of the six yeast strains tested, four strains showed significant enhanced attraction of *A. ervi*, among which *M. reukaufii* evoked the most significant response [$t_{(38)} = 6.512$, $P < 0.001$], followed by the reference strain Y182 [$t_{(38)} = 2.800$, $P = 0.008$], *A. pullulans* [$t_{(38)} = 2.976$, $P = 0.005$], and *M. gruessii* [$t_{(38)} = 2.303$, $P = 0.027$]. By contrast, parasitoid females showed a significant negative response to *S. roseus* [$t_{(38)} = 2.029$, $P = 0.047$], indicating a repellent effect. In addition, no attraction or repellency was recorded for parasitoid females toward *H. uvarum* [$t_{(38)} = -1.656$, $P = 0.106$]. The equal distribution of parasitoids when both odor sources were provided with the control nectar demonstrated that there was no positional bias within our experimental set-up [$t_{(38)} = 0.156$, $P = 0.877$].

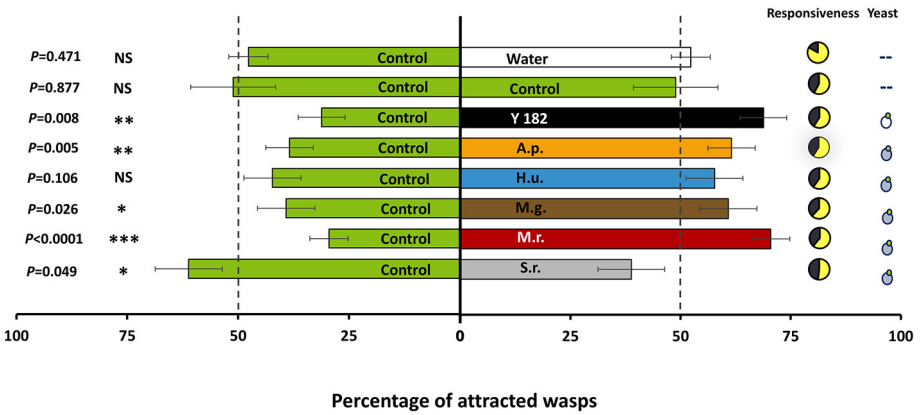

FIGURE 2 | Olfactory response of adult *Aphidius ervi* females when given the choice between two odors (percentage ± SE, $n = 100$). Treatments included: Control, non-inoculated, yeast-free nectar; Water, distilled water; Y182, nectar fermented with the reference strain *Saccharomyces cerevisiae* Y182; A.p., *Aureobasidium pullulans*-fermented nectar; H.u., *Hanseniaspora uvarum*-fermented nectar; M.g., *Metschnikowia gruessii*-fermented nectar; M.r., *Metschnikowia reukaufii*-fermented nectar; S.r., *Sporobolomyces roseus*-fermented nectar. Nectar-inhabiting yeasts are marked with a blue yeast-like symbol, whereas the reference strain is marked with a white yeast-like symbol. Experiments were performed with cell-free nectars. The bioassay was carried out by releasing 20 groups of five females at the base of a two-choice Y-olfactometer and evaluating their response 10 min after their release. Wasps that passed a set line at the end of the olfactometer arms and were still there at the time of evaluation were considered to have chosen for the odor source connected to that olfactometer arm. Parasitoids that did not make a choice within 10 min after release were considered as non-responding individuals, and were excluded from the statistical analysis. Pie charts show the distribution of responding (in yellow) and non-responding (in black) individuals. Asterisks indicate a preference that is significantly different (*t*-test) from a 50:50 distribution within a choice test: ***$P < 0.001$; **$0.001 \leq P < 0.01$; *$0.01 \leq P \leq 0.05$; NS, non-significant.

Additionally, parasitoid females showed similar response to the control treatment or to water [$t_{(38)} = 0.727$, $P = 0.472$; **Figure 2**], indicating that the nectar medium itself has no repellent or attractant effect on the parasitoids.

Impact of Yeast-Fermented Nectars on Nectar Intake, Longevity and Survival

Total nectar consumption (the total amount of nectar consumed measured over a total period of 9 h) significantly differed between nectars [$F_{(6, 28)} = 5.52$, $P < 0.001$]. More specifically, intake of *S. roseus*-fermented nectar was 3-fold less than the control nectar, which was also the case, but to lesser extent, for *A. pullulans*-fermented nectar (**Figure 3**). Similar to nectar intake, yeast-fermented nectars had a significant impact on parasitoid life span [$F_{(6, 28)} = 16.19$; $P < 0.001$; **Figure 4A**] and survival (Log-rank test = 112.54, $df = 6$; $P < 0.001$; **Figure 4B**). Specifically, yeast inoculation significantly reduced parasitoid longevity with 6.6, 7.4, 7.7, and 9.3 days when parasitoids were fed on nectar fermented with Y182, *A. pullulans*, *S. roseus* and *H. uvarum*, respectively (**Figure 4A**). In contrast, no differences in longevity were observed compared to the control when parasitoids were fed on nectar fermented with the nectar specialists *M. gruessii* and *M. reukaufii* (**Figure 4A**).

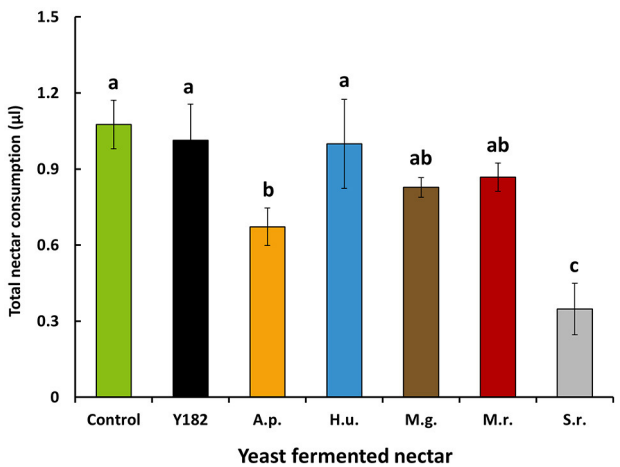

FIGURE 3 | Mean nectar intake (± SE, $n = 5$, each with 15 individuals per cage) by feeding-inexperienced adult *Aphidius ervi* parasitoids after 9 h of nectar supply. Parasitoids were provided different nectars, including: Control, non-inoculated, yeast-free nectar; Y182, nectar fermented with the reference strain *Saccharomyces cerevisiae* Y182; A.p., *Aureobasidium pullulans*-fermented nectar; H.u., *Hanseniaspora uvarum*-fermented nectar; M.g., *Metschnikowia gruessii*-fermented nectar; M.r., *Metschnikowia reukaufii*-fermented nectar; S.r., *Sporobolomyces roseus*-fermented nectar. Experiments were performed using cell-free nectar solutions. Different letters above colored bars indicate significant differences between provided nectars ($P < 0.05$), based on Student-Newman-Keuls method (*F*-test).

DISCUSSION

Here, we demonstrate that specialist, but not generalist, nectar inhabiting yeasts that rely on flower foraging insects for their dispersal produce attractive scent profiles for a generalist aphid parasitoid without affecting its survival and longevity.

Impact on Scent Profiles and Behavioral Response

Our results clearly show that NIYs significantly change the scent profile of nectar and that there was considerable variation

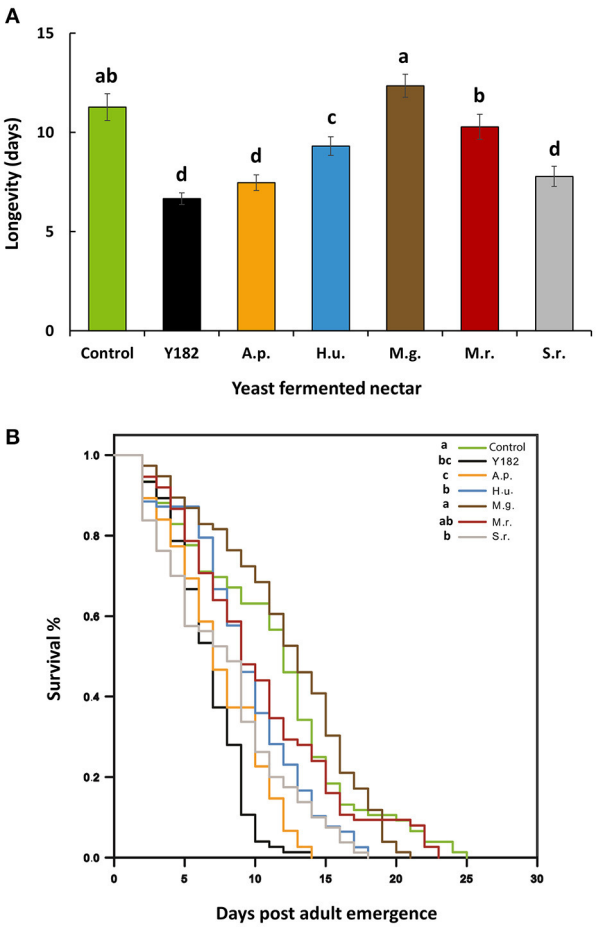

FIGURE 4 | (A) Mean longevity (± SE, $n = 75$, equally distributed over 5 containers) and **(B)** Kaplan–Meier survival curves of *Aphidius ervi* adults fed on different nectars, including: Control, non-inoculated, yeast free nectar; Y182, nectar fermented with the reference strain *Saccharomyces cerevisiae* Y182; A.p., *Aureobasidium pullulans*-fermented nectar; H.u., *Hanseniaspora uvarum*-fermented nectar; M.g., *Metschnikowia gruessii*-fermented nectar; M.r., *Metschnikowia reukaufii*-fermented nectar; S.r., *Sporobolomyces roseus*-fermented nectar. Experiments were performed with cell-free nectar samples under laboratory conditions of 22°C, 70% relative humidity and a 16:8 h light:dark photoperiod. Capillaries with nectar solutions were replaced daily to avoid microbial contamination. Different letters on bars in **(A)** indicate significant differences between provided nectars ($P < 0.05$), based on Student-Newman-Keuls method (F-test). In **(B)** different letters indicate significant differences between curves (pairwise log–rank *post-hoc* tests with Bonferroni correction, $P < 0.05$, $n = 75$).

between yeast species, suggesting that NIYs emit volatile blends that are to a large extent species specific, corroborating earlier findings (Rering et al., 2017). Volatiles produced by NIYs are mainly byproducts or secondary metabolites of the yeast metabolism or fermentation but may have diverse ecological functions (Dzialo et al., 2017). For example, volatile compounds such as ethyl acetate, 2-butanol, isobutanol, ethanol, 2-ethyl-1-hexanol and 2-phenylethanol have been shown to inhibit microbial growth (Cruz et al., 2012; Hua et al., 2014; Pereira et al., 2016), and may help explain why earlier nectar-colonizers often suppress the growth of later arriving microbial species (Peay et al., 2012; Vannette and Fukami, 2014). Furthermore, microbes that rely on insects for dispersal or survival may produce volatiles that are attractive to the insect vectors (Dzialo et al., 2017). For example, compounds like 3-methyl-1-butanol and 2-phenyl ethanol, which are commonly produced by many yeasts including those investigated in this study, are very attractive to a wide diversity of insects (Davis et al., 2013), including hymenopteran insects (Davis et al., 2012; Rering et al., 2017).

Yeasts like *M. gruessii* and *M. reukaufii* are highly abundant nectar specialists (Pozo et al., 2011) that largely rely on floral visitors for dispersal among flowers (Brysch-Herzberg, 2004; Belisle et al., 2012). Furthermore, it was recently found that these specialist yeasts rely on multiple floral visits and repeated inoculations in the nectar to establish their dominant abundance in the nectar microbial community (Mittelbach et al., 2016). Therefore, it is reasonable to expect that these yeasts produce attractive volatiles that aid in their dispersal. Indeed, bumblebees not only responded positively to flowers colonized by *M. reukaufii* (Schaeffer et al., 2014), but also spent significantly longer foraging time on *M. reukaufii*-inoculated flowers compared to yeast-free flowers (Schaeffer et al., 2017). Interestingly, this robust attraction to *Metschnikowia* spp. has also been reported for pest insects (Witzgall et al., 2012). Additionally, *M. reukaufii* has been shown to produce a distinct volatile blend which was the most attractive to honey bees among different microorganisms tested (Rering et al., 2017). In line with these observations, we also found that parasitoid females were attracted the most to *M. reukaufii*-fermented nectar, followed by the reference *S. cerevisiae* strain Y182, *A. pullulans,* and *M. gruessii*. In contrast to *M. gruessii* and *M. reukaufii*, the other tested yeasts (i.e., *A. pullulans*, *H. uvarum* and *S. roseus*) are ubiquitous yeasts that are associated with a wide diversity of habitats, including diverse aerial plant parts (Andrews et al., 1994; Nakase, 2000; Jolly et al., 2014). It can therefore be hypothesized that these yeasts are less dependent on insect vectors or differ in dispersal vectors, and therefore produce different or lower amounts of volatile compounds. Except for the results with *A. pullulans*, which also showed a strong parasitoid attraction, our results support this hypothesis. Indeed, in contrast to the other yeasts tested, both *H. uvarum* and *S. roseus* did not produce high levels of volatiles, and were also not attractive to *Aphidius* parasitoids. *S. roseus* was even found to be deterrent to *A. ervi*. In line with our results, *A. pullulans* has also been previously reported to produce volatile compounds that attract insects (Davis and Landolt, 2013; Hung et al., 2015). The PCA of scent profiles provided further indications on which compounds may be of importance for parasitoid attraction. In particular, isoamyl acetate, isobutyl acetate, 2-methyl propanol, 3-methyl-1-butanol and 2-phenyl ethanol had the greatest loadings for PC1 and PC2, suggesting that production of these compounds correlates most strongly with parasitoid attraction. However, further research with pure chemical compounds is needed to unravel their exact contribution to parasitoid attraction. Moreover, it is not unreasonable to assume that the insect's behavior will depend on blends of these MVOCs, rather than on a single compound, as has been shown for plant volatiles (Takemoto and Takabayashi, 2015).

In addition to volatile compounds that may have an effect on insect behavior, amino acids may also have a notable effect on insect chemoreceptors (Hansen et al., 1998; Carter et al., 2006). In particular, it was found that glutamic acid, leucine and methionine have the potential to modify insect behavior by stimulating insect chemosensory orientation (Wacht et al., 2000). Strikingly, yeast-fermented nectars that showed a non-attractive or repellent response to *A. ervi* females (i.e., *H. uvarum* and *S. roseus*) produced these amino acids in high concentrations, suggesting a potential role for these amino acids in the parasitoids rejection of these nectars. Interestingly, further supporting explanation is provided by the multivariate analysis which disclosed that glutamic acid, leucine and methionine were among the greatest loadings in the first two PCs, highlighting their potential contribution to shape the parasitoid behavior.

Impact on Nectar Chemistry, Nectar Intake, and Survival

Overall, our results show that NIYs strongly affect nectar sugar and amino acids composition and concentration, thereby corroborating previous findings. Interestingly, whereas the different yeast strains depleted several amino acids (arginine, aspartic acid, histidine, serine and threonine) compared to the control nectar, increased concentrations of specific amino acids such as asparagine, alanine, glutamine and methionine were also detected. Moreover, NIYs considerably impacted nectar acidity with a manifest drop in pH from 5.76 to even 3.91 following the inoculation of *A. pullulans*. As a result, it can be expected that such changes in nectar chemistry may impact the overall nectar's appeal and nutritional value (Petanidou, 2005; Nicolson and Thronburg, 2007; Gijbels et al., 2014), thereby potentially also affecting life history parameters such as longevity (Lenaerts et al., 2017). When parasitoids were provided the various yeast-fermented nectars, their nectar intake was distinctly affected. While none of the tested nectars showed enhanced consumption relative to the control nectar, nectars fermented with *S. roseus* and *A. pullulans* were consumed significantly less. One potential explanation for this reduced consumption could be the change in amino acid profile caused by these two yeasts compared to the control (Hendriksma et al., 2014). Overall, *S. roseus* was the only yeast that increased the total amino acid content (**Table 1**). Further, both *S. roseus* and *A. pullulans* increased the amount of aspartic acid and phenylalanine compared to the control. Recently, it has been shown that relatively high concentrations of amino acids such as phenylalanine may inhibit feeding on sucrose solutions containing them, and can act as inhibitors during associative learning (Simcock et al., 2014). This may provide a potential explanation for the reduced consumption of nectar fermented by *S. roseus* and *A. pullulans*. In addition, inoculation with these yeasts resulted in a reduction of pH and a distinct acidity profile. It has been shown that many pollinators avoid acidic nectars (Vannette et al., 2013; Good et al., 2014; Junker et al., 2014). By contrast, other insects such as fruit flies seem to prefer an acidic diet over neutral or alkaline pH food (Deshpande et al., 2015).

In line with nectar intake, NIYs also significantly affected the survival and longevity of the parasitoids. Particularly, parasitoids that fed on *S. roseus*, *A. pullulans* and Y182-fermented nectars showed shortest longevity and lowest survival, suggesting that these nectars lack important nutrients or contain one or more unsuitable compounds. Interestingly, these yeasts consumed more sucrose and simultaneously produced higher amounts of glucose and fructose compared to the other yeasts. Likewise, reduced longevity was observed for *A. ervi* adults fed on nectar inoculated with the bacterium *Asaia* sp. which similarly decreased the sucrose concentration whereas the glucose and fructose content increased (Lenaerts et al., 2017). Similarly, bees and eusocial wasps preferred nectars that contain a high amount of sucrose (Petanidou, 2005). Although sucrose and its hexose components glucose and fructose are considered very suitable carbohydrate sources for most hymenopteran parasitoids (Wäckers, 2001; Luo et al., 2010), further research is needed to find out whether the absolute content of sucrose, glucose and fructose affects nectar consumption and survival of *Aphidius* wasps. By contrast, parasitoid longevity and survival was not affected by inoculation of both *Metschnikowia* species. Similarly, a recent study has shown that *M. reukauffii* had no adverse effects on bumble bee reproduction, including initiation of egg laying and number of eggs laid (Schaeffer et al., 2017).

It has to be noted that we only examined effects of the chemical changes induced by the NIYs by testing cell-free nectar media, while direct effects of the microbes were not considered. It is generally accepted that the microbes themselves can also provide insects with many benefits, e.g., acting as a nutrition source, detoxifying harmful substances, protection from biotic stresses (Crotti et al., 2009; Gibson and Hunter, 2010; Vannette and Fukami, 2016). Further, potential plant effects were not taken into account. In this regard, it may be possible that the effects observed in this study may be different from those seen in field studies or in-flower inoculations, as plants may also influence nectar chemistry (Canto et al., 2017; Vannette and Fukami, 2018).

Potential Applications

Recently, there is an increasing interest in harnessing insect-microbe chemical communications to control insect pests in agricultural systems (Davis et al., 2013; Beck and Vannette, 2017). In particular, it has been shown that MVOCs produced by yeasts robustly mediate host finding and food location for a wide range of insects (Dzialo et al., 2017), including sap beetles (Nout and Bartelt, 1998), codling moth (Witzgall et al., 2012), spotted wing drosophila (Scheidler et al., 2015; Mori et al., 2017), European grapevine moth (Tasin et al., 2011) and coffee bean weevil (Yang et al., 2017). Interestingly, these findings promote the possibility of exploiting yeast-based attraction as an ecofriendly technique to control pest insects, e.g., by luring them away from the crop or attract and kill them using specific traps (Davis and Landolt, 2013; Andreadis et al., 2015). Based on our results, a similar strategy could be developed to attract natural enemies into the field and prevent pest populations from reaching the economic injury level. In this regard, further study should focus on the specificity of the interactions to ensure only beneficial insects are attracted.

CONCLUSIONS

Overall, our results indicate that nectar yeasts modulate floral nectar attractiveness to flower-visiting insects by producing distinctive scent profiles. Furthermore, we have demonstrated that feeding on these fermented nectars affected insect longevity and survival. Interestingly, our results support the hypothesis that microorganisms that almost solely occur in nectar and that are therefore strongly dependent on floral visitors for dispersal produced volatile compounds that enhance insect attraction. Additionally, we showed that these microorganisms had no adverse effects on the longevity and survival of their vectors. Nevertheless, the exact consequences of altered insect behavior for the yeasts, the insects, and also the plants, still remain unclear to date and requires further study. Additionally, we only examined responses of the generalist parasitoid *A. ervi,* so it is possible that other flower-visiting insects respond differently. Our results also provide support to recent suggestions that secondary metabolites signaling between yeasts and insects can be used as a promising tool for sustainable crop protection, e.g., to improve methods currently used in controlling or monitoring insect pests (Beck and Vannette, 2017). Further research is needed to investigate the feasibility of such strategy.

AUTHOR CONTRIBUTIONS

ISS, HJ, and BL conceived the ideas and designed the experiments. ISS, DB, TG, BH-M, and LB performed the experiments and collected the data. ISS, DB, HJ, and BL analyzed the data. FW and KV contributed to equipment, reagents and materials. BH-M and WV contributed to nectar chemical analysis. ISS, HJ, and BL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. The authors have declared that no competing interests exist.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01009/full#supplementary-material

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Biochemical Traits in the Flower Lifetime of a Mexican Mistletoe Parasitizing Mesquite Biomass

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Psittacanthus calyculatus is a hemiparasitic plant that infects a wide range of trees. Mainly the biology reproduction of this mistletoe lies in bright colored flower development. Furthermore, it uses the nectar secretion as the only reward to engage different flower visitors. We investigated the physiological mechanisms of the flower phenology per hour and per day to analyze the spatial-temporal patterns of the nectar secretion, Cell Wall Invertase Activity (key enzyme in the quality of nectar), nectar chemistry, volatile organic compounds (VOCs) emission, synthesis of carotenoids and frequency of floral visitors. Flowers lasted 4 days, total nectar was loaded just before the anthesis and the secretion was maintained over day 1 and 2, decreased on day 3, and stopped on day 4. The diurnal nectar secretion dynamic per hour on day 1 and 2 showed similar patterns with high production on the morning and a decrease in the afternoon, the secretion declined on day 3 and ceased on day 4. On the other hand, CWIN activity per day was less before the anthesis and increased on day 1 and 2, this enzymatic activity decreased on the old flower phenology. Moreover, diurnal CWIN activities showed different patterns in the morning, noon, and lastly in the afternoon. Nectar chemistry varied significantly throughout of the flower lifetime, sucrose decreased along the flower phenology increasing glucose and fructose. Amino acids showed the prevalence of proline and oxo-proline, both increased on the day 1 and diminished in subsequent old flower stages. The spatial VOCs emission showed the presence of 11 compounds being β-ocimene the main volatile; its release increased on day 1 and remained constant in the flower lifetime. Lutein, lycopene, and β-carotene were concentrated in old stages of the flowers. In field, the most frequent flower visitors were the hummingbirds that usually foraging in all phenologic flower stage and their foraging events decreased with the phenological flower lifetimes. The results showed that these traits presented by *P. calyculatus* flowers are able to engage and manipulate the behavior of flower visitors and contribute to the reproduction of the parasitic plant.

Keywords: floral phenology, volatile organic compounds, nectar chemistry, floral cell wall invertase, carotenoids

INTRODUCTION

The attraction of pollinators by flowers is based on different traits such as: floral nectar (FN) secretion, volatile organic compounds (VOCs) emission, and the production of color compounds in the flowers, these features are called pollination syndromes (Knudsen and Tollsten, 1993; Faegri and Van der Pijl, 2013). These pollination syndromes, as well as floral longevity and phenology are involved in the pollinator attraction behavior and floral specialization (Fenster et al., 2004; Guerra et al., 2014). Highly rewarding plants are common in a community with high diversity and they produce unique signals to ensure pollination (Schiestl and Johnson, 2013; Dar et al., 2017). These signals encourage pollinators to establish recurrent visits on flowers of these species, leading into fitness advantages in terms of increased receipt and export of intraspecific pollen to pollinate different flowers that results in the reproduction of plants (Wright and Schiestl, 2009; Rosas-Guerrero et al., 2014).

Flowering plants used different strategies to produce showy colored flowers with high secretion of FN (Zimmerman, 1988; Lucas-Barbosa, 2016). The latter is used as a unique reward to "manipulate" the pollinator behavior during and immediately following plant visits, affecting positively the pollen transfer and therefore plant reproduction (Aukema, 2003; Janovský et al., 2017). These plants offer high amounts of FN secretion (Ramírez and Ornelas, 2010) rich in sucrose and amino acids, which is biochemical adapted to the pollinator attraction (Heil, 2011). Furthermore, high quality nectar synthesis requires a complex enzymatic machinery. The first Arabidopsis flowers' gene that encodes an apoplastic Cell Wall Invertase (CWIN) has been reported, this gene is compulsory to upload sucrose from the phloem and catalyze the hydrolysis of sucrose into glucose and fructose in nectar solution (Ruhlmann et al., 2010). Also, CWIN is important in the partition of sucrose in the extrafloral nectar of *Acacia cornigera* (Orona-Tamayo et al., 2013) and *Ricinus communis* (Millán-Cañongo et al., 2014). The FN quality increases when it contains different amino acid concentrations such as proline, an energetic amino acid common in FNs, this amino acid is involved in the flying maintenance of insects and hummingbirds (Carter et al., 2006; Nepi et al., 2012). A classic example of interactions between the flowering plants that include these traits to attract pollinators is the well-known interaction between hummingbirds with mistletoe plants. Mistletoes comprise an aerial parasitic plants composed of around of 1,500–1,600 species worldwide (Nickrent et al., 2010). They are present in a variety of forms and are exclusively found in the tropical native species of South and Central America, Africa, Australia, and New Zealand (Fadini et al., 2018). These plants usually present different traits to manipulate the pollinator behavior that include: extended flowering lifetime (Azpeitia and Lara, 2006), *production of high quality FN* (Rivera et al., 1996; Pérez-Crespo et al., 2016), *VOCs emission* (Bungert et al., 2002; Sipes et al., 2014) and the developments of bright colored flowers (Pérez-Crespo et al., 2016). Among mistletoes the Loranthaceae family is one of the largest and the most diverse (73 genera and ∼990 species) (Nickrent et al., 2010).

The genus *Psittacanthus* is one of the most spectacular parasitic plants (∼120 species) that distributed from Mexico to the northern of Argentina (Kuijt, 2009). These mistletoes are found in 25 Mexican states situated in the central and southern regions (Azpeitia and Lara, 2006). Some of mistletoe species are totally dependent on bird pollination for their reproduction as reported for *P. calyculatus* (Azpeitia and Lara, 2006), *P. shiedeanus* (Ramírez and Ornelas, 2010), *P. robustus* (Guerra et al., 2014) and *P. auriculatus* (Pérez-Crespo et al., 2016) all of them show the syndrome of ornithophilus species. *Psittacanthus calyculatus* (DC.) G. Don (Loranthaceae) is an American mistletoe commonly found from Mexico to Venezuela (Azpeitia and Lara, 2006). In the central region of Mexico, this mistletoe parasitizes mainly mesquite (*Prosopis laevigata*) an endemic tree of this region. This parasite has become one of the main menace to the existence and death of mesquites, because the tree does not resist living with the mistletoe, however, a kill mesquite is used as biomass to produce different co-products.

We used *Psittacanthus calyculatus* that parasitizing *Prosopis laevigata* biomass to evaluate the floral lifetime and used this phenology to evaluate the spatial-temporal patterns of the nectar secretion, CWIN activity in the nectar secretion, nectar chemistry, VOCs emission, synthesis of carotenoids and frequency of floral visitors to link the flower traits with a strategy of mistletoe reproduction.

MATERIALS AND METHODS

Study Area and Plant Material

The experiments were performed in a population of *Prosopis laevigata* (mesquite) highly infected with *P. calyculatus* located in a suburban area near of Irapuato in the state of Guanajuato in Central Mexico (20°43′ N; 101°19′ O at 1,730 m a.s.l). The weather of the area is mainly mild and humid, but dry at the end of each year. The rainy season is present in summer, with a mean annual precipitation of 650 mm and temperature of 18°C. All plants and flowers used showed no visible signs of infection by phytopathogens or damage by herbivores. Nectar quantification, and collection of flowers were conducted from June to September of 2015. Material collected to analyze the CWIN activities, nectar chemistry (sugars and amino acid compositions), carotenoid content, and floral visitor's quantification were performed in 2016.

Floral Phenology

We built up a categorization in order to classify the phenology. For this experiment, floral buds were selected in 10 mistletoe plants. The floral longevity and petal color were recorded during 5 days after petal flower excision. A semi-open bud was classified as "day 0" (D0), and subsequently, flower opening widely was designed as day 1 (D1), early young stages were designed as day 2 (D2) and day 3 (D3), mature stages were recorded as day 4 (D4), however, on day 5 (D5) petals had fallen and this

stage was not taken for experiments. Only active flowers with nectar secretions were selected to analyze on the subsequent experiments.

Temporal Patterns in Floral Nectar Secretions

The experiments were based on Azpeitia and Lara (2006) with modifications. Prior to the FN quantification, close and semi-open buds including the leaves were placed inside mesh bags to avoid floral visitors and they only were removed as soon as the nectar was quantified. Nectar was extracted from buds and flowers were measured at intervals 07:00, 09:00, 11:00, 13:00, 15:00 and 17:00 h during five consecutive days without removing the flowers. In a separate experiment, FN was collected at 07:00 during five consecutive days. FN was removed using a micropipette of 20 μl of volume, and the concentration of soluble solids was quantified with a temperature-compensated hand refractometer (Atago Co., Japan) as described earlier (Heil, 2004; Orona-Tamayo et al., 2013; Millán-Cañongo et al., 2014). To recover and record the nectar volume on the refractometer the nectar was collected using 5 μl microcapillaries. Rate secretions were calculated separately from the different stages. In all cases, different flower stages were collected after last collection of FN and oven-dried at 60°C to relate the FN amounts (as soluble solids) to the dry mass of the secreting flower by hour.

Determination of Sugars and Amino Acids From the Floral Nectar Secretion

Nectar was collected and pooled from 10 plants and stored in a 1.5 ml in water-ice, after the nectar collection this was immediately frozen and stored at −70°C until further analysis. For the analysis of free sugar and amino acids we followed the methods described by Pais et al. (1986) with some modifications. For sugars, 10 mg of nectar was lyophilized, resuspended with 1 ml ultra-pure water, and the solution was passed through a cationic exchange Dowex 50w-x8 column (Bio-Rad, Hercules, CA, United States). Column was washed four times with 1 ml water and the aqueous solution containing sugars were collected and evaporated to dryness in a rotator evaporator. The amino acids retained in resin were eluted with the addition of four times of 1 ml of 4 M NH_4OH. The solution was collected and processed as described earlier.

Nectar sample compounds were processed by reaction with addition of 20 μl pyridine and 80 μl *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), the mixture was incubated for 30 min at 80°C. After this time, 1 μl of each sample were analyzed by a gas chromatography system (Agilent 7890A; Agilent Technologies, Santa Clara, CA, United States) coupled to a mass-selective detector (Agilent 5975C; Agilent Technologies, Santa Clara, CA, United States) with a capillary column (60 m × 250 μm × 0.25 μm coating; Agilent Technologies, Santa Clara, CA, United States). Helium was used as carrier gas with a flux of 1 ml/min and the following temperature program was used: initial temperature at 70°C for 5 min and ramped at 5°C/min until 310°C for 15 min. The initial temperature of the injector was of 250°C. Carbohydrate and amino acids standards were prepared using the same methodology. Sugar and amino acid were identified using the National Institute of the Standards and Technology version 2.0 (NIST).

Cell Wall Invertase Activity on the Floral Nectar Secretion

The floral nectaries are found in the base of the calyx of the flower of *P. calyculatus* (Galetto et al., 1990), and it has been reported that in these structures occur the presence of the CWIN (EC 3.2.1.26) which is a key enzyme involved in the quality, production and responsible for the hexose-rich composition of the FN (Ruhlmann et al., 2010). CWIN activity from the floral nectaries of *P. calyculatus* was determined in two independent experiments, one resembling the conditions as in the experiment designed to determine the time course in FN secretion each 2 h, and the other one resembling of FN each 24-h. All experimental conditions were as mentioned above. Only flower calyx that contains the floral nectary tissues were collected and pooled from 10 randomly selected plants for each experiment and finally they were immediately frozen in dry ice. Other flower parts were discarded (Azpeitia and Lara, 2006). Enzymatic activity was quantified as described by Orona-Tamayo et al. (2013) and Millán-Cañongo et al. (2014) with some minor modifications. Ground tissue (25 mg) was mixed with 5 mg of polyvinylpyrrolidone (PVP) and then with 500 μl of ice-cold 50 mM HEPES-NaOH (pH 8.0, containing 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$ and 1 mM $CaCl_2$). Samples were incubated on ice for 10 min and then centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was discarded and the pellet containing the cell walls associated invertases was washed three times with 500 μl of extraction buffer by re-suspending and centrifugation as described above. Finally, pellets were washed with 500 μl of ice-cold 80 mM sodium citrate, pH 4.8 and the invertase activity was measured as described previously (Orona-Tamayo et al., 2013; Millán-Cañongo et al., 2014) with some modifications. In short, 300 μl of 80 mM sodium citrate (pH 4.8; room temperature) were added to the pellets and the mixture was incubated at 37°C. The tubes were then centrifuged at 10,000 × *g* for 1 min at room temperature and 20 μl of each sample was mixed with 180 μl of HK reaction solution [Glucose (HK) Assay Kit Product Code GAHK-20, Sigma-Aldrich]. After reaching the steady state, 100 μl of an aqueous 100 mM solution of sucrose was added to the samples and the absorption was immediately measured at 340 nm in a μQuant® Microplate-reader continuously. Aliquots were taken every 20 min for 80 min and analyzed as before.

VOCs Emitted From the Floral Phenology

Volatile collections were performed in the different phenological flower stages that were mentioned above. However, due to asynchronous development of the *P. calyculatus* flowers, bunches only presented one, two or three stages that precluded the analysis of VOCs *in situ*. We collected and pooled 10 flower per stage and were placed into a 50-ml Erlenmeyer flask with tap water (1 cm of deep) and immediately were enclosed

with aluminum foil and Parafilm®, and the VOCs were adsorbed using a Solid Phase Micro-Extraction (SPME; 2 cm, carboxen/Polydimethylsiloxane/Carbowax; Supelco, Bellefonte, PA, United States). Fibers were exposed by a period of 6 h. After this time, fibers were desorbed for 30 s into the GC-MS, and the program temperatures for separation were as follows: 60°C through 80°C at 5°C/min; 210°C at 8°C/min maintained at 210°C for 5 min (Quintana-Rodriguez et al., 2015). VOCs were identified using the NIST library.

Carotenoids Accumulation in Flower Phenology

Determination of carotenoids in petals from the floral phenology were determined according to the methods of Li and Beta (2012) with some modifications. Petals (10 flowers) were detached and calyx and anthers were discarded, these were frozen and grinding. Samples were protected from the light and lyophilized.

To determine the total floral carotenoid contents, all manipulations were performed under dim light to avoid the minimal photochemical degradation. Tissue (0.1 g of petals) were extracted using 1 ml of mixture of ethanol (100%) and 0.1% butylated hydroxytoluene (BHT), the mixture was transferred to a Recti-Vial (Pierce Co.), mixed and incubated by 10 min at 80°C. After this time, 20 μl of 20% KOH was added and mixture was incubated as before. The mixture was combined with 500 μl of hexane (100%) and 1 ml of water and samples were centrifuged at 12,000 × *g* for 5 min at 4°C. The supernatant was collected and the remained residue was re-extracted as before until the residue was colorless and supernatants were combined. Extraction solvent was combined with 50 μl of extract and the absorption was measured at 470 nm in a microplate format. Total carotenoids were calculated using the following equation and expressed as μg/g (Li and Beta, 2012).

$$\text{Total carotenoids } (\mu\,g/g) = (Ab^{*}V^{*}10^{6})/(A^{1\%}{}^{*}100G)$$

Ab is the absorbance at 470 nm, V is the total volume of extract, $A^{1\%}$ is the extinction coefficient for a 1% mixture of carotenoids at 2500 and G is the sample in dry weight (g).

For carotenoid composition, the carotenoid extracts were separated and quantified on Ultra-High Performance Liquid Chromatography (UHPLC) (Agilent 1200 infinity LC systems, Agilent Technologies, Santa Clara, CA, United States) coupled with a photodiode array detector was used. Carotenoids were separated using a Zorbax Eclipse Plus C-18 column (2.1 mm× 5.0 mm, 1.8 μm) with a temperature maintained at 40°C. The separation was achieved by a main solvent composition as followed: 55% methanol/40% acetonitrile/5% dichloromethane/0.1% BHT. The solvents were filtered through 0.45 μm membrane. The system was run in isocratic mode with a flow rate was kept constant at 0.4 ml/min for a total run time of 10 min. The injection volume of each sample and standards was 1 μl and absorbance was measured at 450 nm. The identification of the carotenoids was based on the congruence of retention times with those of pure carotenoids standards.

Floral Visitors Related to the Phenology Stages

The frequency of flower visitors on the floral phenology was based on methods reported by Azpeitia and Lara (2006) and Guerra et al. (2014) with modifications. Open-buds, young and old flowers from different bunches (10 plants) were pruned-off. Only closed-buds were placed into mesh bags and then labeled. As soon as the buds were open (D0), we begin our records, which actually started from 6:30 h to 17:00 h by five consecutive days. We performed 80 h of focal observations over different days (10 days) and they were performed by using binoculars (10-22x50; Nikon) at distances between 10 and 15 m from the plants.

Data Analysis

Data were analyzed with Least Significant Difference (LSD) *post hoc* tests after analysis of variance (ANOVA) due they meet the assumptions of heterogeneity and homoscedasticity and normal distribution, and in the case of visitors frequency in flowers, we used a χ^2-test to evaluate the similarity in the visitor frequency and were performed using the Statistical Package for the Social Sciences 17.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Time-Course of Floral Phenology

Psittacanthus calyculatus flowers had a lifespan of 5 days, on the fifth day petals had fallen totally (**Figure 1**). In addition, petals showed a remarkable change of color through their phenologic stages. On D0, buds were partially open (semi-open bud) on the tip and presented a light yellow coloration, similarly color presented on D1, this color persisted on D2, while on D3 through D4 petals turned on a bright orange and on the D5 the petals fallen completely.

Patterns of Floral Nectar Secretion

Psittacanthus calyculatus flowers produced high volume of nectar as a main pollinator's reward. FN secretion per day collected at 24-h showed a high concentration of nectar in opened-buds (D0; 1.09 mg g^{-1} h^{-1} dm) collected at 07:00 am before the flower aperture (**Figure 2A**); this high nectar trend continued at

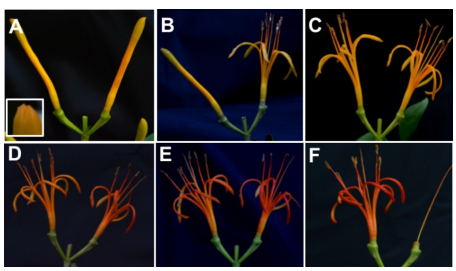

FIGURE 1 | Time-course of flower stages. **(A)** Semi-opened bud on day 0 (D0) with an apical aperture on the tip. **(B)** The beginning of the flower anthesis on day 1 (D1); **(C)** day 2 (D2); **(D)** change of color on day 3 (D3); **(E)** day 4 (D4); **(F)** day 5 (D5).

the beginning of anthesis in the phenologic stage D1 (1.06 mg g^{-1} h^{-1} dm) and was maintained on the D2 (1.05 mg g^{-1} h^{-1} dm). However, the nectar secretion decreased on the D3 (0.35 mg g^{-1} h^{-1} dm) and dropped with the flower age on the D4 (0.04 mg g^{-1} h^{-1} dm) ($P < 0.05$) (**Figure 2A**).

The diurnal spatial patterns of nectar secretion collected each 2-h varied significantly ($P < 0.05$) in D1 (07:00–17:00) with concentrations of 1.1–0.55 mg g^{-1} h^{-1} dm, and increased again from 13:00 h (0.96 mg g^{-1} h^{-1} dm), and finally it decreased between 15:00 and 17:00 (**Figure 2B**). While on D2, the spatial nectar secretion was similar in amount and behavior to D1, FN was presented in high rate in the morning (07:00; 1,2 mg g^{-1} h^{-1} dm), decreased at 09:00 (0.70 mg g^{-1} h^{-1} dm), increased at the next times recorded (11:00–13:00; 1.0 mg g^{-1} h^{-1} dm) and showed a significant decrease at 15:00 (0.68 mg g^{-1} h^{-1} dm) and at 17:00 (0.64 mg g^{-1} h^{-1} dm) ($P < 0.05$). On D3 the nectar secretion showed a decrease in all times recorded: in the morning (07:00; 0.56 mg g^{-1} h^{-1} dm) presented a nectar reduction than the other days ($P < 0.05$), in the next points measured (09:00–11:00; 0.24–0.35 mg g^{-1} h^{-1} dm) it was more evident the nectar reduction. A dramatic nectar dropped was presented on D4 in all hours recorded, however in the morning (07:00; 0.001 mg g^{-1} h^{-1} dm) a significant nectar secretion rate ($P < 0.05$) was showed, but at followed hours the nectar was stopped completely (**Figure 2B**). In summary, the high nectar secretion rate was presented in the morning on the first 3 days.

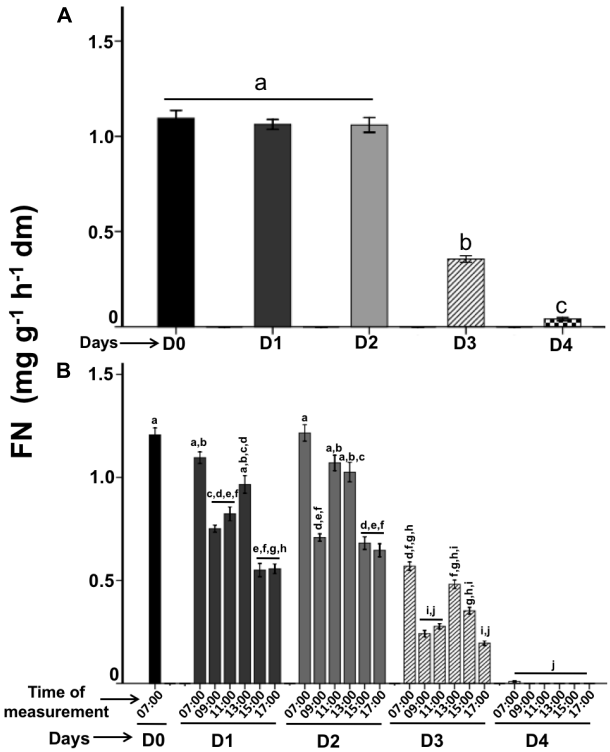

FIGURE 2 | Dynamics of floral nectar secretion during the flower lifetime. **(A)** Floral nectar secretion was quantified each 24-h. **(B)** Diurnal floral nectar secretion measured each 2-h per day. Bars represent means ± SE ($N = 66$) of FN secretion (mg soluble solids per g flower dry mass and hour).

Patterns of Cell Wall Invertase Activity

Cell wall invertase is a β-fructofuranosidase that catalyzes the hydrolysis of sucrose into glucose and fructose (Roitsch and González, 2004). It has been suggested that this enzyme plays an important role in the nectar secretion (Ruhlmann et al., 2010; Orona-Tamayo et al., 2013; Millán-Cañongo et al., 2014). We used the above described FN patterns secreted per day and diurnal hours to investigate whether CWIN activity in floral nectaries are the main responsible for the FN secretion.

Interestingly, on the phenological stages per day, we observed low enzymatic activity in opened-buds (D0; 2.07 μg glu ml^{-1} min^{-1}; $P < 0.05$). However, when the flower anthesis begun, we found a CWIN high activity level between D1 and D2 (3.9 and 4.2 μg glu ml^{-1} min^{-1}, respectively) (**Figure 3A**) that is related with the main peak of FN secretions (**Figure 2A**). On D3, CWIN displayed low activity (2.8 μg glu ml^{-1} min^{-1}), on day D4 CWIN activity decreased (1.6 μg glu ml^{-1} min^{-1}; $P < 0.005$). Therefore, the temporal patterns in the CWIN activities preceded the pattern in FN behavior by 2 h and these activities diminished due to FN display a reduction on the same phenological stages (**Figures 2A**, **3A**).

The CWIN activity (recorded each 2-h) varied significantly in diurnal hours. The D0 stage (07:00) showed a low enzymatic activity of 1.8 μg glu ml^{-1} min^{-1} (**Figure 3B**). However; CWIN activity increased on the next day D1 when the anthesis began; at 09:00 (4.0 μg glu ml^{-1} min^{-1}) the highest activity on this day was recorded at 11:00 (3.7 μg glu ml^{-1} min^{-1}), and it decreased gradually from 13:00 to 17:00 (2.5–0.9 μg glu ml^{-1} min^{-1}). On D2, CWIN activity displayed similar activities to D1, in the morning (07:00) the activity was low (2.0 μg glu ml^{-1} min^{-1}), then it increased at 09:00 (1.5 μg glu ml^{-1} min^{-1}), and the highest activity was registered at 11:00 (3.7 μg glu ml^{-1} min^{-1}), then it decreased gradually from 13:00 to 17:00 (2.5–0.9 μg glu ml^{-1} min^{-1}). CWIN activities diminished on D3–D4 showing similar low enzymatic activities at 07:00 (1.9 and 1.2 μg glu ml^{-1} min^{-1}, respectively), which gradually decreased from 09:00 to 15:00 (1.5 μg glu ml^{-1} min^{-1}) to finally dropped to its lowest at 17:00 (1.0 and 0.75 μg glu ml^{-1} min^{-1}; respectively). The CWIN activities per hour were more actives on D1-D2 than D3–D4, when these activities diminished, in parallel the nectar dropped, and the flower aged.

Nectar Chemistry on Flower Lifetime

Nectar sugar concentration varied throughout the flower lifetime. We found that sucrose was the dominant sugar followed by glucose and fructose both present in a similar concentration. Whereas sucrose concentration decreased gradually, glucose and fructose increased lightly as the flower age (**Figure 4**). Therefore, sucrose concentration was higher on D0 (83.0%; $P < 0.05$) than on the other days and glucose (10.5%) and fructose (6.5%) showed lower concentration. When the anthesis began on D1, sucrose decrease (77.2%) and glucose (13.6%) and fructose (9.22%) increase lightly. Similarly, on D2 the sucrose dropped (69.3%) and hexoses increased (glucose: 16.7%; fructose: 13.9%) and sugar reductions were more evident on D3 (sucrose:

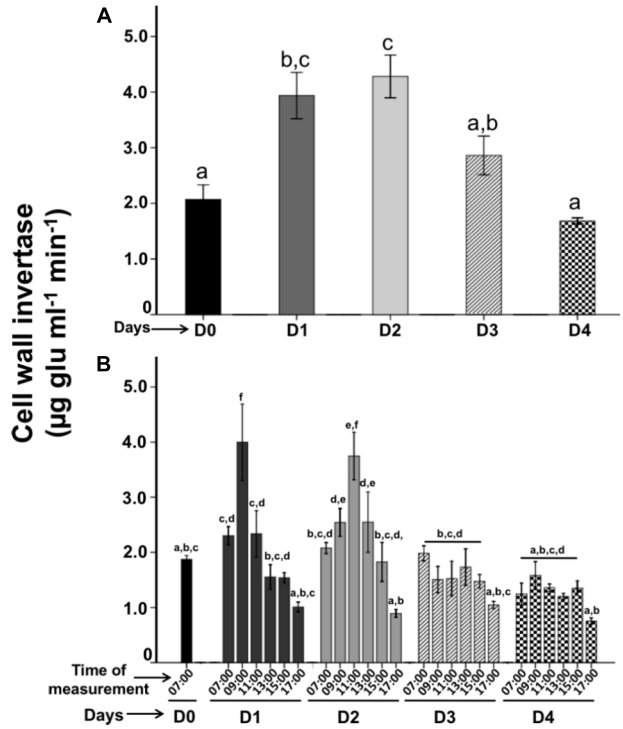

FIGURE 3 | Time-course of Cell wall invertase (CWIN) activities during the active floral nectar secretion on the flower lifetime. **(A)** CWIN activities were quantified each 24-h during the active floral nectar secretion. **(B)** Diurnal CWIN activities were quantified during the floral nectar secretion measured each 2-h. Bars represent means ± SE (*N* = 3) of sucrose hydrolyzing activity (µg glucose released per min per ml).

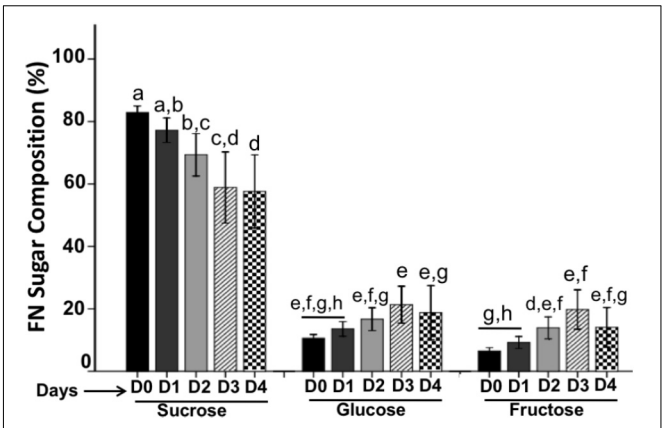

FIGURE 4 | Sugar composition in floral nectar. FN is composed by sucrose as the main sugar followed by glucose and fructose. Bars represent means ± SE (*N* = 8) and reported as %.

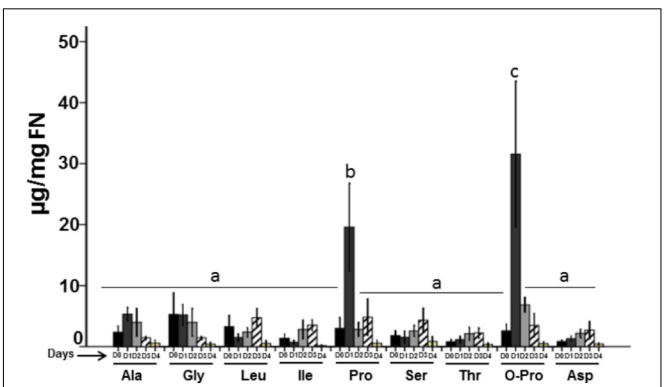

FIGURE 5 | Total amino acids in floral nectar. The FN is composed by different amino acids, however, oxo-proline and proline were the most abundant. Bars represent means ± SE (*N* = 8) and are reported as µg/mg of nectar.

58.8%) a showing increase of glucose (21.3%) and fructose (19.7%). Finally, the concentration of sucrose dropped on the D4 (57.6%), and hexoses decreased (glucose: 18.7%; fructose: 14.0%).

Nectar from the four flower stages studied (**Figure 5**) had a significant concentration of amino acids. We found alanine (Ala), glycine (Gly), leucine (Leu), isoleucine (Ile), proline (Pro), serine (Ser), threonine (Thr), oxo-proline (O-Pro), and aspartic acid (Asp). On D0 these amino acids showed similar concentration, however; on the next day (D1), Pro and O-Pro presented a higher concentration (0.19 and 0.31 µg/mg FN, respectively; $P < 0.05$), while the rest of amino acids did not increased their concentration ($P < 0.05$). On the D2, Pro (0.07 µg/mg FN) and O-Pro (0.03 µg/mg FN) showed a high reduction in their concentration. On the days D3–D4, Pro and O-Pro, as well as all amino acids, showed a more pronounced reduction.

Volatile Organic Compounds Profiles From Flower Lifetime

In the floral VOCs profile, we identified 11 compounds from the *P. calyculatus* flower stages, and these compounds showed significant qualitative differences (**Table 1**). All of those compounds were present on the DO stage. However, only eight of those volatiles were present in the subsequent flower stages (see compounds in **Table 1**). On D2 six volatiles were present, while, on D3 only five compounds were collected and finally on D4 only two VOCs were identified. β-Ocimene was the main volatile presented through the flower lifetime; on D0, this volatile showed a value of 58.6% and increased on D1 (71.0%), on D2 this compound showed the highest concentration (94.2%) that dropped lightly (88.0%) on D3. The VOC 2,4-di-tert-butylphenol (DTBP) increased on the stage D1 (12.8%) and decreased on day D2 (2.1%) and disappeared on flower stage D3. Two compounds were emitted constitutively in all flower stages (β-ocimene and geranyl nitrile), while nonanol and β-farnesene were present only on stages D0 and D1 and disappeared in subsequent flower stages.

Total Content of Carotenoid Composition on Flower Phenology

The total carotenoids composition throughout the flower lifetime is shown in **Figure 6A**. On the stage D0 carotenoids content was lowest with a value of 259.7 µg/g dm, but this concentration increased and these values reached a maximum values on the

TABLE 1 | VOCs emitted from the flower lifetime.

	Days				
Compound	**D0**	**D1**	**D2**	**D3**	**D4**
Cis-β-ocimene*	58.6 ± 19.5b	71.0 ± 22.7b	94.2 ± 4.9a	87.9 ± 3.9b	94.8 ± 1.6a
Geranyl nitrile	9.0 ± 3.8b	5.2 ± 2.7b	2.2 ± 0.9a	6.9 ± 2.5a	2.6 ± 1.2a
1-hepten-4-ol	3.5 ± 1.8a	4.6 ± 1.9b	2.4 ± 1.6a	3.0 ± 1.1a	ND
Cis-hexenyl isovalerate	2.2 ± 0.8	ND	ND	ND	ND
Cis-3-hexenyl acetate	2.8 ± 1.2b	3.1 ± 2.0ab	2.3 ± 1.2a	1.4 ± 0.2b	ND
3-hydroxy-2,2,4-trimethyl pentyl ester of isobutanoic acid	5.9 ± 3.7	ND	ND	ND	ND
Cis-hexenyl butyrate	4.2 ± 2.4	ND	ND	ND	ND
Nonanal*	2.1 ± 1.0a	2.8 ± 1.8a	ND	ND	ND
β-farnesene*	2.9 ± 0.3a	2.7 ± 1.8b	ND	ND	ND
2,4-bis-(1,1-dimethylethyl)-6-methyl phenol	9.4 ± 1.3ab	12.8 ± 14.4ab	2.1 ± 1.7a	ND	ND
Neryl acetate	2.4 ± 1.3ab	1.3 ± 0.3ab	1.7 ± 2.0a	1.7 ± 0.6a	ND

*Compounds in the table are ordered according to their retention time. Total peak area percentage per gram of fresh weight of normalized five replicates are expressed in the table. Different letters represent statistically differences for each compound per day ($p < 0.05$ according post hoc Tukey's HSD after ANOVA). ND, not detected. *Compounds identified with commercial standards.*

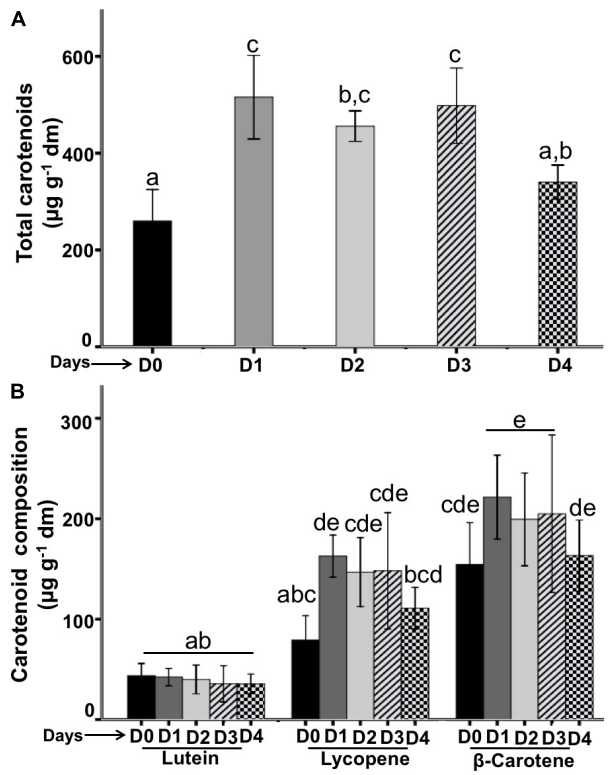

FIGURE 6 | Quantification of carotenoids from flowers during the flower lifetime. **(A)** Total carotenoids contents were quantified each 24-h. **(B)** Composition of carotenoid types during the flower lifetime. Bars represent means ± SE ($N = 6$ for separately experiments) of carotenoids concentrations (µg per g flower dry mass).

next days D1–D3 (515.7–498.1 µg/g) and this concentration significantly dropped on D4 (340.1 µg/g).

Carotenoid composition on the flower phenology was composed by lutein, lycopene and β-carotene (**Figure 6B**). Lutein was the compound with the lowest concentration on all flower stages (D0–D4) that ranged from 43.7 to 35.6 µg/g. Lycopene was the second less concentrated compound in the flower lifetime, this compound on day D0 was present in a lowest concentration (79.1 µg/g) and increased on D1 (162.8 µg/g), its concentration was similar on the D2 and D3 (146.8–148.2 µg/g, respectively); and decreased on D4 (110.8 µg/g). β-carotene was the main compound presented in the flower lifetime, on D0 showed the lowest concentration (154.4 µg/g) that was increased on D1 (221.5 µg/g) and the concentration was similar on the subsequent days D2 and D3 (199.4–204.9 µg/g, respectively), but on D4 the concentration decreased significantly (110.4 µg/g).

Visitors on the Flower Lifetime

To understand the relationship between flower phenology and visitors, we used the *in situ* phenology and recorded only four visitors: hummingbirds, bees, butterflies and wasps (**Figure 7**) that have been documented to be the regular visitors of *P. calyculatus* (Guerra et al., 2014; Pérez-Crespo et al., 2016). The main visitor in the different phenological flower stages were hummingbirds followed by bees, butterflies, and wasp (**Figure 8**). On day D0 we did not register visitors to search nectar, however on day D1, we recorded a total of 52 hummingbirds foraging events and their visits peaked actively between 09:00 and 15:00 h; the second visitors recorded were bees (25 foraging events) that actively visited flowers between 11:00 and 13:00 h, in both cases the visitors diminished at 17:00 h; butterflies were the third visitors (12 foraging events) with active hour from 11:00 to 13:00 and decreased at 17:00; wasps were occasional visitors (two foraging events) at 09:00, and between 13:00 and 15:00 h. On D2, we observed 28 foraging events by hummingbirds, beginning at 07:00, with its highest abundance peak at 09:00 and decreased on the following hours. Bees (15 foraging events) were more active between 11:00 and 15:00 h. While, butterflies (five foraging events) showed the highest abundance at 11:00 h and decreased at 13:00 h, and wasps visits (two foraging events) were occasional. On D3, all floral visitors diminish in its foraging events, we

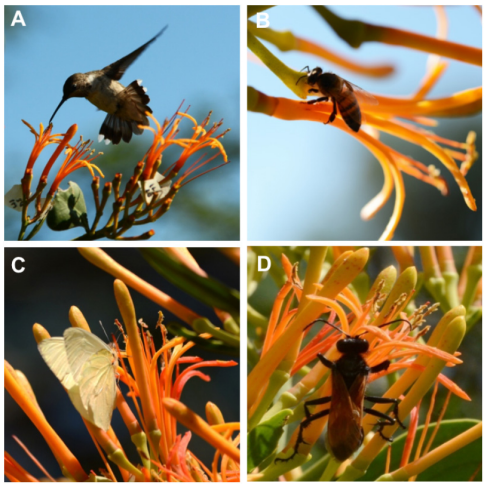

FIGURE 7 | *P. calyculatus* visitors during different lifetime. **(A)** A *Cynanthus latirostris* hummingbird flying on flower on day 1. **(B)** *Apis mellifera* robbing floral nectar on day 2. **(C)** *Ascia* sp. butterfly accessing to the tube that contained floral nectar. **(D)** *Pepsis* sp. foraging behind of the mistletoe flower.

recorded that hummingbirds showed only 15 foraging events with a similar behavior on the hours recorded. However, we recorded an abundance on bee (nine visits), and butterflies only presented two foraging events, and an absence of wasps on all hour recorded. On D4, we observed a dramatically foraging events in all floral visitors, hummingbirds once again presented 10 foraging events showing its highest abundance peak at 07:00 h; bees visited this stage with only six visits with maximum abundance at 11:00 h, butterflies represent (four visits) being more abundant at 11:00 h, and finally we observed that the less abundant visitor were wasps (less of one foraging events). We note that all foraging events dropped in parallel with the phenological flower stages and flower visitors diminished their visits to the old flowers.

DISCUSSION

Flower Lifetime of *P. calyculatus*

Flower phenology of *P. calyculatus* lasted 5 days of metabolic activity; in anthesis, the color changed from a light yellow, which persisted from D0 to D2. However, we observed a color change between D2 to D3 as a bright orange color, and on D4 petals changed totally to a bright-red; finally, on D5 petals had fallen. Similar results were found by Azpeitia and Lara (2006); however, they recorded 1 day more of floral longevity rather than our study. Other mistletoe flowers of the Loranthaceae family, showed different phenology stages such as *P. shiedeanus which* lasted 6 days (Ramírez and Ornelas, 2010), *Ligaria cuneifolia* lasted 4 days (Rivera et al., 1996), *P. robustus* lasted 3 days (Guerra et al., 2014) and *P. auriculatus* lasted 2 days (Pérez-Crespo et al., 2016). These differences can be due to the regional zones that presented environment changes that involve a high genetic divergence between mistletoe species.

Floral Nectar Patterns on the Flower Lifetime

The dynamic of FN secretion from flowers in anthesis showed different amounts of nectar from semi-open bud (D0) through the old flower stages. Nectar secretion is loaded at the bud stage (D0), in this stage the bud is loaded with a high concentration of nectar; this active secretion continues for 48-h and decreased in old flower phenologic stage on D3 and become zero on D4. Azpeitia and Lara (2006) presented quantitative information on the FN secretion in *P. calyculatus*; they found that these flowers secreted high amount of nectar on the first 3 days and diminished in the last phenologic flower day, similar to our results. The high concentration of nectar in bud stage can be maintained due to a ready amount of nectar available for the consumption of floral visitors at the beginning of the anthesis. This could indicate an energy saving in the synthesis of nectar per day, which is highly expensive for the plant.

Patterns on Cell-Wall Invertase Activity on the Flower Nectar Secretion

The spatial-temporal patterns of CWIN enzymatic activities resembles those of FN secretion; CWIN activity showed a low activity on D0 and become the highest peak on D1 and D2, this activity dropped on D3 and diminished close to zero on D4. Furthermore, the CWIN activities were similar across the diurnal secretion of nectar by 2-h. These CWIN activities are related to the replenishment of the nectar by day and hour. The FN of *P. calyculatus* contained high concentration of sucrose, but is would be expected the presence of a high hexose concentration; however, CWIN alone cannot be responsible for the differences in hexoses concentration and therefore other enzymes seem to play important roles in determining the FN sugar composition (Ruhlmann et al., 2010). Plants possess other types of invertases isoenzymes such as vacuolar invertase, neutral invertase and cell wall invertases. Alternatively, sucrose unloaded in the sink cells can be cleaved in the cytosol by neutral invertases or by vacuolar invertases, the hexoses resulting, by the activities of sucrose-cleaving enzymes can be used as substrates for different metabolic process (Roitsch and González, 2004). For example, vacuolar and neutral invertases activities seems to have a small influence on the hexose production and concentration regard in *Nicotiana attenuata* nectar (Tiedge and Lohaus, 2018). We do not discard these invertase activities and their influence in the metabolic modulation of sucrose in the nectary tissue of *P. calyculatus* flowers.

In fact, the sucrose came from the phloem, the CWIN enzyme can hydrolyze the sucrose to release glucose and fructose into the FN (Vassilyev, 2010); a general mechanism of nectar secretion could consist of the unloading of sucrose from the phloem via CWIN[1] (Heil, 2015) and/or its synthesis in the floral nectary parenchyma with the aid of sucrose phosphate synthase and sucrose synthase, followed by its secretion into the extracellular space via SWEET9 and then its partial hydrolysis by an apoplastic invertase, which is eventually secreted into the liquid nectar (Heil,

[1]http://www.youtube.com/watch?v=Nd8ryN_7BP8

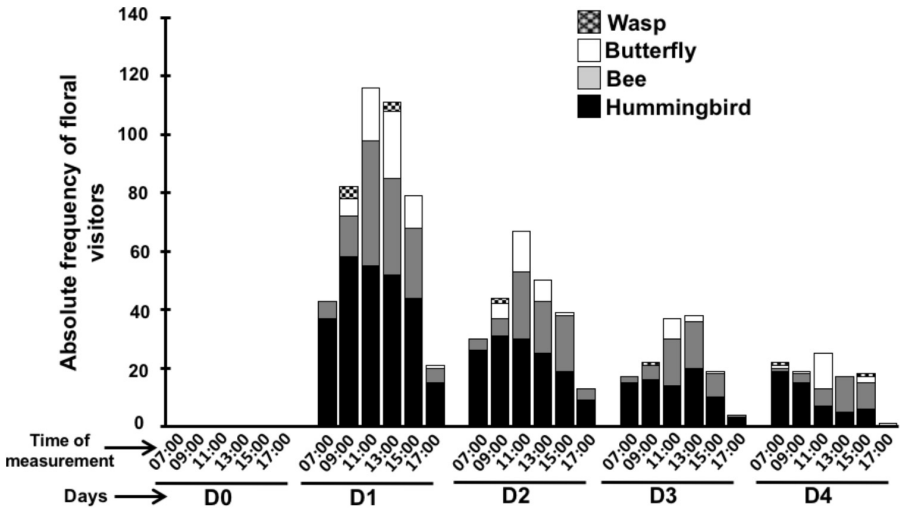

FIGURE 8 | Visitors frequency in flower lifetime. Different consumer of floral nectar measured each 2-h during the flower lifetime.

2015). For example, in *Arabidopsis thaliana* mutant plants that lack CWIN gene resulted in a high sucrose concentration and a lower ratio of hexoses compared to the wild-type ecotype. In fact, in extrafloral nectaries of *Acacia cornigera* CWIN is active in previous hours of nectar secretion and diminishes its activity when nectar cease (Orona-Tamayo et al., 2013), similar CWIN activities occurred in extrafloral nectaries of *Ricinus communis* (Millán-Cañongo et al., 2014). Therefore, this enzyme is an important factor required for the nectar production.

Nectar Chemistry on the Flower Lifetime

Chemical composition in nectar varies significantly throughout of the flower lifetime. There is a constant decrease in the sucrose concentration, however; glucose and fructose increased lightly as the flower phenology. Sugar composition of FN of *P. calyculatus* could be related with CWIN invertase activities that hydrolyze the sucrose releasing hexoses into the nectar. In bud stage (D0) we found a low CWIN activity and a high concentration of sucrose and lower concentrations of glucose and fructose. On the next days, the CWIN activities increased and the sucrose was hydrolyzed and then the hexoses were released into the nectar. This different sugar concentration found in FN in young flower stages could be due to the metabolic machinery rearrange; among the nectar constituent sugars are the most important, because they are the basis of the energy reward to different flower visitors (Baker and Baker, 1983) and this nectar sugar composition is related to the sucrose-cleaving enzymes. Sucrose is more attractive to pollinator birds and insects because these organisms prefer this sugar instead than monosaccharides, for example for hummingbirds, butterflies and other long-tongued bees usually prefer sucrose-rich FNs (Heil, 2011). In the case of FN of *P. calyculatus* flowers, the sucrose is the highest sugar present in all phenology stages this could be a pollination strategy for manipulate the attraction of flower visitors. In *Ligaria cuneifolia,* the sucrose diminished and hexoses increased with respect to flower lifetime (Galetto et al., 1990) similar than our results.

Nectar sugars are present between 100 and 1,000 times more than amino acids, and these can significantly affect the attractiveness of nectar (Heil, 2011, 2015). Certain amino acids are found frequently in different FNs (Baker and Baker, 1986) such as alanine, serine, proline, glycine, isoleucine, and threonine (Baker and Baker, 1973); all of these amino acids were found in *P. calyculatus* FN together with leucine, aspartic acid and oxo-proline. Proline and oxo-proline increased to a high concentration when the flower opened and decreased on the next days, this could be a strategy for pollinator attraction by the tasty nectar amino acids and sugars secreted by the flower. Proline is a normal constituent of many nectars and has also been identified at high levels in plant nectars and its function is due this amino acid can stimulate the insect's salt cell concentration which results in an enhanced feeding behavior (Escalante-Pérez et al., 2012). Studies testing the feeding preference of forager honeybees for proline-, serine-, and alanine-enriched nectars, reported that proline-enriched nectar were preferred by these insects (Bertazzini et al., 2010; Noutsos et al., 2015). The presence of oxo-proline in nectar is a particular case, their presence at least is part of the different amino acid concentrations that are dissolved in the nectar solution; however, their function is involved in the glutathione metabolism, an important antioxidant found in plants, animals, and microorganisms (Gong et al., 2018), and their main function is to prevent the cellular oxidation caused by the reactive oxygen species such as free radicals and peroxides (Sabetta et al., 2017). Moreover, flight effort has been shown to increase oxidative stress levels in birds and insects (Janske et al., 2011) and probably when they seek nectar and consume it, their stress oxidative can be decreased mediated by the oxo-proline that promotes the glutathione synthesis a strong antioxidant in the muscles of the floral visitors. The presence of glucose, proline and oxo-proline in nectar represent a dual action, first proline is for rapid, short-term bursts of energy production and a

large amount of glucose for extended flight (Carter et al., 2006) and oxo-proline an intermediate of glutathione that promotes antioxidant effects.

VOCs Emitted on the Flower Lifetime

Flowering plants use a broad spectrum of signals to attract pollinators some of them are bright colors, and shapes to VOCs (Raguso, 2009). The VOCs profiles found in *P. calyculatus* revealed that the emission of these compounds tended to diminish through the time, being D0 where more compounds were found. Other studies showed that few compounds were found in the VOCs profile of plants pollinated mainly by hummingbirds (Cronk and Ojeda, 2008; Klahre et al., 2011). It has been reported that geranyl nitrile is emitted in petals, stamens, and calyxes of *Robinia pseudoacacia*, for this reason; we suggest that this compound remained present along the flower lifetime, since it can be emitted by distinct parts of the flower (Aronne et al., 2014). Interestingly, many VOCs were released on D0, several of these compounds are related with defensive functions and probably this is their main function in the bud and flowers of *P. calyculatus* (Schiestl, 2010). VOCs such as *cis*-3-hexenyl acetate, *cis*-3-hexenyl isovalerate, *cis*-3-hexenyl butyrate, and nonanal are compounds emitted commonly in response to bacterial or fungi diseases and they play a role as antimicrobial compounds (Pichersky and Gershenzon, 2002; Yi et al., 2009; Heil and Karban, 2010; Quintana-Rodriguez et al., 2015). β-Ocimene is a compound present in all floral phenology, it is a very common volatile released in flowers and has been reported to play multiple functions from the attraction of floral visitors to defensive functions (Farré-Armengol et al., 2017). Is important to note that the floral consumers such as insects, birds, and bats can transfer microflora among flowers, and other plant organs (Fridman et al., 2012), bacteria, fungi, and yeast in nectar may affect the nectar's chemical composition, and thus reduced the pollination success (Vannette et al., 2013). Those volatiles emitted by *P. calyculatus* flower could be exerts an antimicrobial function rather than volatiles involved in the insect attraction or repellence. Future studies will allow determining if many of these compounds have antimicrobial activities.

Carotenoids From the Flower Stages

Carotenoids are responsible for the yellow and red color of flowers and these compounds were changing in the flowering of the *P. calyculatus*. On the initial stages of the bud and anthesis, the flowers showed a yellow color on D0–D2, but on D3 the flowers presented a bright orange color and D4 they turned into a light red color. We can observe that on D2–D3 occur a transition change of yellow light to orange bright color and the flower become an orange intense as the amount of pigment increases; however, our carotenoids quantification did not fit with these flower color changes, only β-carotene the main pigment was constant in their concentration. Carotenoids are a large family of pigments, and are responsible for many of the brilliant red, orange, and yellow color in flowers (Delgado-Vargas et al., 2000). In addition, flowers can contain at least other important group of pigments such as anthocyanins, these are involved in the red color of the flowers (Miller et al., 2011), and we do not discard that these pigments are involved in the flower color transition of light to orange the flower of *P. calyculatus*. Azpeitia and Lara (2006) found similar flowering color pattern to our results, however, pigment petals did not were analyzed at the different floral stages. Color from floral parts constitutes the major visual attractants for pollinators (Ram and Mathur, 1984). When *P. calyculatus* flowers change in color after opening, the nature and biogenesis of floral carotenoids and their quantitative concentrations differ at different flower stages. The carotenoid contents that we found were lutein, lycopene, and β-carotene, lutein was the less concentrated at all flower stages; lycopene increased their concentration from D1 to D3 and diminished on D4. A similar behavior was observed for β-carotene, with a high concentration on D0, but a lower content of this pigment on D4. These patterns of carotenoids degradation can be due to the effect of pollinated flower (Ohmiya, 2013). Ram and Mathur (1984) evaluated the color changes subsequent to the anthesis and determined that the pollination was a key factor as a trigger for a rapid carotenoids and anthocyanin synthesis in the flower lifetime; for this reason, we can observed a carotenoid diminished in the lifetime of *P. calyculatus* flowers. Hummingbirds use their vision principally in finding yellow or bright red color flowers with copious quantities of FN (Miller et al., 2011). Schemske and Bradshaw (1999) found a weak correlation between hummingbird and bee visitors and the color of Monkey flowers (*Mimulus lewissi* and *M. cardinalis*.). In this research, hummingbirds did not exhibited preferences by flowers with similar concentrations of carotenoids or anthocyanins, and petal carotenoids significantly decrease the bee visitations, without effect on hummingbirds, concluding that the high concentration of these pigments function primarily to discourage bee visitation. However, in our field's results we quantified a high percentage of bee visitation, these could be due to the effect of different VOCs that attract these insects. The ability of hummingbirds to quickly find rich nectar sources and to return to them suggests that hummingbirds are capable of exerting strong selection on the nectar rewards of flowers (Schemske and Bradshaw, 1999). These results are consistent with our findings due to the fact that hummingbirds do not have an innate preference for *P. calyculatus* yellow or red flowers and their attraction can be due to the high volumes of nectar in these flowers.

Flower Visitors on the Flower Lifetime

Mistletoes of the Loranthaceae family are strongly associated with ornithophilia syndrome due to the flower morphology (tubular structures, colorful, robust corollas, and resistant sexual organs) (Faegri and Van der Pijl, 2013). We found that the *P. calyculatus* flowers received four types of floral visitors, hummingbirds, butterflies, bees and wasps. Hummingbirds were the main visitors, followed by bees, butterflies, and wasps. The foraging events of hummingbirds begin in the early hours of the morning and these are gradually reduced at midday; however, we found interestingly that as the visits of hummingbirds decreased, the visits of the insects increased, this could be to avoid conflicts of negative interactions between the nectar

consumption. In addition, those foraging visits decreased in the different phenological flower stages and could be a direct effect of the nectar cease. Previous studies carried out on *P. calyculatus* found that its flowers are visited by four species of hummingbirds, observing a greater presence of visits also in the mornings and decreasing at the midday (Azpeitia and Lara, 2006) similar to our observations. The presence of hummingbirds as main pollinators in other mistletoe species such as *P. schiedeanus* (Ramírez and Ornelas, 2010), *P. robustus* (Guerra et al., 2014), and *P. auriculatus* (Pérez-Crespo et al., 2016) has been observed and therefore they have been cataloged as the main pollinators of these mistletoes, since they describe the hummingbird as an effective carrier of pollen (López de Buen and Ornelas, 1999). The hummingbirds' head dimensions make them ideal for touching the anthers that contain the pollen, they carried the pollen on the head thus when visiting another flower, they can fertilize it. On the other hand, insects have a tiny size and they do not have effective contact with the anthers and pollination is not performed (Azpeitia and Lara, 2006). The presence of hummingbirds is related to the extended phenological flowering is seen as a pattern in mistletoes pollinated by these birds (Galetto et al., 1990; Rivera et al., 1996). It has been observed in different mistletoes that having high nectar production and flower longevity rates suggest a greater attraction of pollinators (Ramírez and Ornelas, 2010). The number of pollinator visits can be influenced by a large number of factors such as environmental conditions (temperature and humidity), attractions (visual and volatiles), and rewards (nectar and pollen) signals (Proctor et al., 1996). The plant ensures the attraction of pollinators through the anthesis of the flower by secreting large amounts of nectar rich in components such as sugars and amino acids that pollinator organisms seek to maintain their biological daily activities (Guerra et al., 2014). In that aspect, the hummingbird is possibly attracted by the sweet taste of the nectar derived from sucrose mainly, as well as by proline and oxo-proline.

In sum, the flowers of *P. calyculatus* contain different traits such as nectar production enriched with an excellent quality of biomolecules such as sucrose, glucose, and fructose, and energetic amino acids such as proline and oxo-proline as a reward for pollinators. The quality of sugar is due to the CWIN activity that biochemically is synchronized with the nectar secretion on the flower lifetime, the lower emission of VOCs was involved in the insect attraction, antimicrobial effects, or insect repellence, the showy color flower is composed by different carotenoids with functions of attraction and repellence. This is the first time that different traits are evaluated in this mistletoe flowers. These important suits can enforce pollination specificity and manipulate the behavior to improve the pollination in flowers of *P. calyculatus*.

AUTHOR CONTRIBUTIONS

EQ-R and DO-T designed the study, performed the experiments, and wrote the manuscript. AR-R and ER-C performed the experiments. XC-C performed the statistical tests. JM-T and JE-C provided equipment. MH provided intellectual suggestions.

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Reduced Responsiveness to Volatile Signals Creates a Modular Reward Provisioning in an Obligate Food-for-Protection Mutualism

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Plants in more than 100 families secrete extrafloral nectar (EFN) to establish food-for-protection mutualisms with ants. Facultative ant-plants secrete EFN as a jasmonic acid (JA)-dependent response to attract generalist ants. In contrast, obligate ant-plants like the Central American "Swollen-Thorn Acacias" are colonized by specialized ants, although an individual host can carry ant colonies from different species that differ in the degree of protection they provide. We hypothesized that hosts that associate simultaneously with various partners should produce rewards in a modular manner to preferentially reward high quality partners. To test this hypothesis, we applied JA to distinct leaves and quantified cell wall invertase activity (CWIN; a regulator of nectar secretion) and EFN secretion by these "local" (i.e., treated) and the "systemic" (i.e., non-treated) leaves of the same branch. Both CWIN activity and EFN secretion increased in local and systemic leaves of the facultative ant-plant *Acacia cochliacantha*, but only in the local leaves of the obligate ant-plant, *A. cornigera*. The systemic EFN secretion in *A. cochliacantha* was associated with an enhanced emission of volatile organic compounds (VOCs). Such VOCs function as "external signals" that control systemic defense responses in diverse plant species. Indeed, the headspace of JA-treated branches of *A. cochliacantha* induced EFN secretion in both plant species, whereas the headspace of *A. cornigera* caused no detectable induction effect. Analyses of the headspace using GC-MS identified six VOCs in the headspace of *A. cochliacantha* that were not emitted by *A. cornigera*. Among these VOCs, β-caryophyllene and (*cis*)-hexenyl isovalerate have already been reported in other plant species to induce defense traits, including EFN secretion. Our observations underline the importance of VOCs as systemic within-plant signals and show that the modular rewarding in *A. cornigera* is likely to result from a reduced emission of the systemic signal, rather than from a reduced responsiveness to the signal. We suggest that modular rewarding allows hosts to restrict the metabolic investment to specific partners and to efficiently sanction potential exploiters.

Keywords: exclusive rewards, extrafloral nectar, obligate mutualism, sanctions, systemic signaling, volatile signals

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INTRODUCTION

Most mutualisms are formed by hosts that interact with multiple partners. Partners can differ in their quality as mutualists, and non-reciprocating partners infer a cost to their host without providing the corresponding benefit (Sachs, 2015). Therefore, theory predicts the evolution of "host sanctions" or other mechanisms that allow hosts to adjust reward provisioning to the quality of the service they receive (Bshary and Grutter, 2002; Kiers et al., 2003; Kiers and Denison, 2008). Host sanctions have been reported for mutualisms such as the legume–rhizobia mutualism, in which plants were reported to "penalize" non N-fixing nodules (Kiers et al., 2003; Westhoek et al., 2017), or for the fig–fig wasp mutualism, in which the fig tree aborted figs that were colonized by non-pollinating wasps (Jandér et al., 2012). Intriguingly, Kiers et al. (2003) and Jandér et al. (2012) observed sanctions to occur in a modular manner: only nodules that did not fix nitrogen or figs that were carrying non-cooperative wasps were sanctioned. Evidently, a modular provisioning of rewards is adaptive in symbiotic systems in which different parts of the same host are colonized simultaneously by different partners. In contrast, hosts that engage in facultative mutualisms with non-symbiotic partners should provide rewards in a more systemic way, in order to enhance their attractiveness to mutualists that eventually visit the host (Agrawal and Rutter, 1998).

A modular reward provisioning has been reported from symbiotic mutualisms whereas systemic reward production characterizes common facultative mutualisms, but the molecular pathways remain poorly understood that enable plants to allocate rewards in different spatial patterns. Here, we use extrafloral nectar (EFN) secretion to identify the mechanism that controls the modular *versus* a more systemic production of a reward in an ant-plant mutualism. Extrafloral nectar (EFN) is produced by plants from more than 4,000 species in ca. 750 genera (Weber and Keeler, 2013) to attract ants and other predators, or even parasitoids, all of which act as an indirect defense against herbivores (Heil, 2015). The main components of EFN are mono- and disaccharides and amino acids, but proteins are also frequently reported (Escalante-Pérez and Heil, 2012). The content of metabolically costly compounds and the observation that EFN secretion can be limited by light availability (Bixenmann et al., 2011; Jones and Koptur, 2015) indicate that EFN is a costly reward whose production should be under strict control by the plant. Most EFN-secreting plant species function as facultative ant plants, i.e., they secrete EFN in response to herbivory as a jasmonic acid (JA)-dependent defense mechanism to establish facultative mutualisms with generalist ants, which are attracted from the vicinity (Heil et al., 2001; Roy et al., 2017). The inducibility of EFN secretion by JA finds its mechanistic explanation in the fact that cell-wall invertase (CWIN), which represents a central limiting step in the secretion of nectar (Ruhlmann et al., 2010; Lin et al., 2014; Roy et al., 2017), is induced by JA (Millán-Cañongo et al., 2014). In contrast, so-called myrmecophytes, which have been described from over 100 genera of plants, provide nesting space–and usually also a food reward such as EFN - to colonies of symbiotic "plant-ants". These interactions are considered obligate mutualisms, because the plant-ants depend on their host for food and nesting space, whereas the plants depend on the ants for protection (Heil and McKey, 2003). In simple words, facultative ant-plants recruit generalist ants from the vicinity when defense is actually required, whereas obligate ant-plants provide nesting space and food to a "standing army" of specialized ants (**Figure 1**).

In the present study, we used two EFN-secreting plant species from the same genus to test the hypothesis that the obligate ant-plant provides this reward in a modular way whereas the facultative ant-plant provides the reward systemically, and to identify the molecular mechanism that allows for a modular versus systemic reward production. Obligate ant-plants such as the Mesoamerican "Swollen-thorn Acacias" (*sensu* Janzen, 1974), such as *A. cornigera*, *A. hindsiii* and *A. collinsii*, offer EFN and cellular food bodies as food rewards for obligate plant-ants from the *Pseudomyrmex ferrugineus* group (Janzen, 1966; Ward, 1993; Seigler and Ebinger, 1995; Ward and Branstetter, 2017). These ants colonize their host partly, or completely, and protect the colonized parts from herbivores, climbers and pathogens (Janzen, 1967, 1969; González-Teuber et al., 2014; see **Figure 2**, and Supplementary Video File 1). In contrast, other species such as *A. farnesiana*, *A. cochliacantha* and *A. macracantha* (Seigler and Ebinger, 1988) engage in facultative mutualisms with generalist ants species that patrol these plants (Bentley, 1977; Tilman, 1978; Koptur, 1992; Agrawal, 1998). In the latter three plant species, EFN secretion has already been shown to be induced by damage or the exogenous application of JA (Heil et al., 2004). Besides herbivory, mechanical damage, or treatment with JA, EFN secretion can also be induced by volatile organic compounds (VOCs), at least in lima bean (*Phaseolus lunatus*) (Kost and Heil, 2006; Heil and Silva- Bueno, 2007). In fact, VOCs have been reported as external signals that orchestrate systemic responses to local attack in diverse plant species, comprising both monocots and dicots (Frost et al., 2008; Heil and Ton, 2008; Heil and Karban, 2010; Schrader et al., 2017). Considering this role of VOCs, we hypothesized that a modular versus systemic production of an inducible reward like EFN could result from differences in the emission of plant VOCs or in the responsiveness of the plant to these VOCs (**Figure 1**). In short, in this study, we employ a comparative approach to investigate whether plant VOCs can act as airborne plant hormones that generate different spatial patterns in the EFN secretion by a facultative and an obligate ant-plant.

MATERIALS AND METHODS

Plant Species and Study Site

The plant species used in this study were *Acacia cornigera* (L.) Willdenow, an obligate ant-plant, and *Acacia cochliacantha* Humb. Bonpl. ex Willd., a facultative ant-plant. All plants selected for this study were shrubs 1–2.5 m tall growing at their natural site in in the coastal area in Southern Mexico close to Puerto Escondido, Oaxaca (∼15°55′N and 097°09′W). Plant species were determined following Janzen, 1974 and Seigler and Ebinger (1988, 1995), and ant species were determined following (Ward, 1993; Ward and Branstetter, 2017), and confirmed by P.S. Ward. Due to the polyphyly of the former genus *Acacia* s.l.

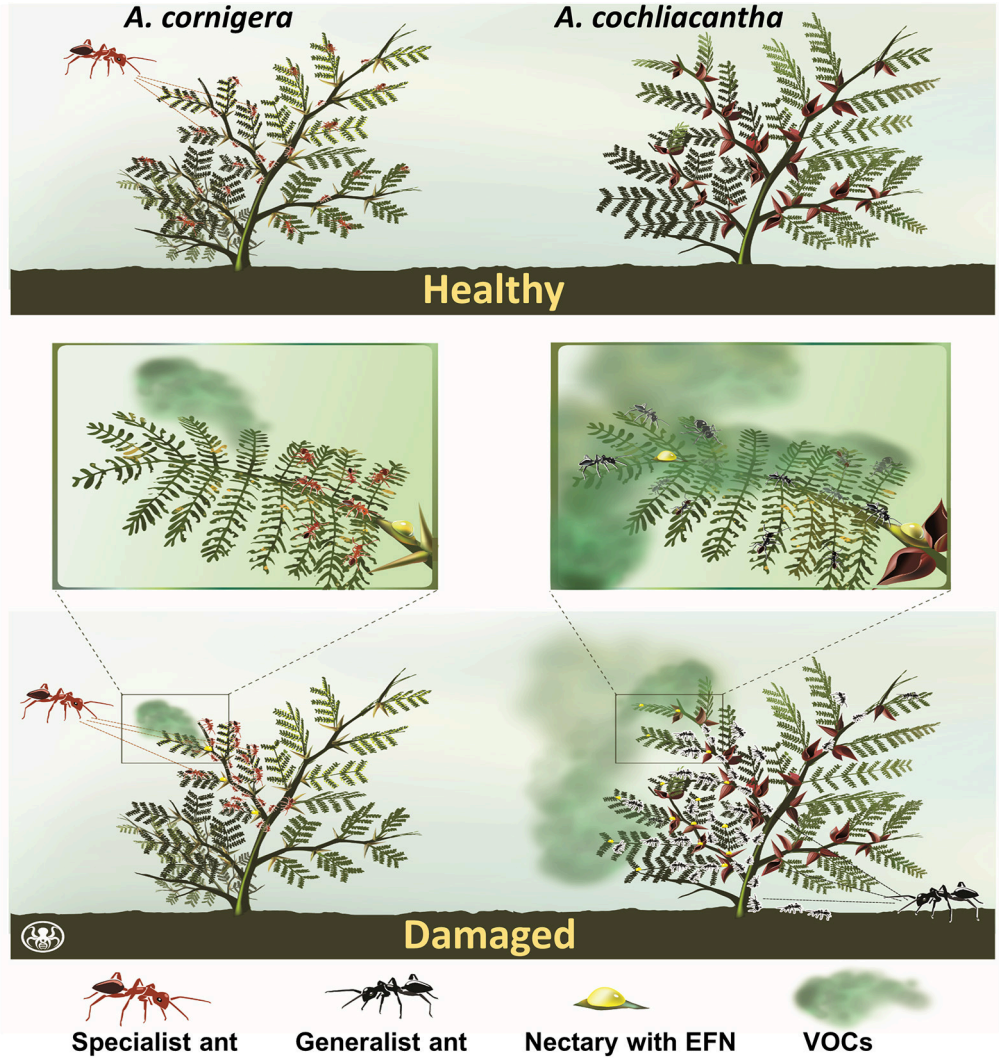

FIGURE 1 | Study species and hypothesis. The obligate ant-plant, *A. cornigera* and the facultative ant-plant, *A. cochliacantha*, are hypothesized to respond differently to local damage. *A. cornigera* is colonized by a standing army of specialist ants (*P. ferrugineus*) even in the absence of damage ("healthy", upper panel). These ants are quickly recruited when the plant is locally damaged (lower panel), a response that can be elicited by plant VOCs (Agrawal, 1998; Inui and Itioka, 2007; Mayer et al., 2008; Schatz et al., 2009) or by a strongly modular induction of extrafloral nectar (EFN) secretion (this study). In contrast, *A. cochliacantha* responds to damage with the attraction of generalist ants from the vicinity, a situation in which the number of ants recruited should correlate positively with the amount of EFN secreted. Since VOCs can control systemic plant responses (Heil and Ton, 2008), a modular versus systemic reward production by the obligate vs. the facultative ant-plant might be explained by differences in the emission of—or the response to—such VOCs.

it has been recommended to term the Mesoamerican clade of the former genus *Acacia* "*Vachellia*" (Orchard and Maslin, 2003; The Legume Phylogeny Working Group, 2017), a suggestion that has been discussed intensively (Luckow et al., 2005; Smith and Figueiredo, 2011; Kyalangalilwa et al., 2013). For the sake of reproducibility and in order to allow comparisons with published work, we respect the iconic term "Swollen thorn Acacias" as introduced by Janzen in 1974 and, hereinafter, use the species names as defined in the beforementioned taxonomic keys, which have been used to identify our study species.

Effect of JA and Ants on EFN Secretion

Earlier observations suggested that obligate *Acacia* ant-plants secrete EFN constitutively (Heil et al., 2004). However, only plants that were colonized by the obligate plant-ant, *P. ferrugineus*, had been used in that study, which tempted us to hypothesize a role of the ants in EFN secretion. In order to investigate the effects of ants and of exogenous JA application on EFN secretion, we selected each eight plants of *A. cornigera* and of *A. cochliacantha*; all *A. cornigera* plants were inhabited naturally by *P. ferrugineus* ants, whereas the *A. cochliacantha* plants were visited by generalist ants such as *Camponotus*

FIGURE 2 | Modular colonization and defence of an *A. cornigera* plant. Obligate myrmecophytes can carry small ant colonies that protect only the colonized branches (white arrows) whereas the remaining parts of the plant remains free of ants and, thus, without a protection from herbivores and pathogens (red arrows). This photo is a part of Supplementary Video File 1. See video file for close-ups.

truncatus, *Crematogaster larrea, Pseudomyrmex gracilis* (species kindly determined by P.S. Ward). From each plant, we selected three branches that were similar in terms of leaf number and age of the branch, and free of visible damage. All branches possessed at least ten healthy leaves, which were numbered according to their age (leaf 1 being the youngest one, **Figure 3**). One branch per plant served as positive control, i.e., it remained inhabited by *P. ferrugineus* ants (*A. cornigera*) or with continuous access for generalist ants (*A. cochliacantha*). The other two branches were deprived of ants as described earlier (Heil et al., 2004). In short, all thorns were cut off (*A. cornigera* only) and all ants were removed from the branch. Then, a ring of Tangle Trap® (a sticky resin that prevents ants from passing, The Tangle Foot Company http://www.planetnatural.com/product/tree-tanglefoot-insect-barrier/) was applied around the base of the branch and finally, the branch was covered with a gauze bag to protect the EFN from flying nectar robbers and the leaves from herbivores (Heil et al., 2004). After 2 days, nectaries on one of these ant-free branches were treated with 20 μL of 1 mM aqueous solution of JA pipetted directly on each nectary or, as a negative control, with 20 μL of Milli-Q® water. Then, all three branches were deprived of ants and protected from nectar consumers as described above and in Heil et al. (2004). The volume and concentration (in equivalents of sucrose) of the secreted EFN was quantified 24 h later with microcapillaries and a portable refractometer (Atago® hand refractometer) for three leaves per branch. Consecutively, these leaves were collected and dried to express EFN secretion as amounts of soluble solids secreted per g of leaf dry mass and 24 h, as described earlier (Heil et al., 2004).

Modular vs. Systemic Response to JA

In order to evaluate whether the two Acacia species investigated here secrete EFN as a modular or a systemic response, we selected eight plants each of *A. cornigera* and *A. cochliacantha* as described above and selected two branches per plant, using the same criteria as above but making sure that each branch had at least ten fully expanded, healthy leaves. One branch per plant was treated by applying 20 μL of 1 mM of aqueous JA solution to the nectaries of leaves 1, 5, and 10, whereas 20 μL of Milli-Q® water was applied to the same nectaries on the control branches. After 24 h, EFN secretion was quantified as described above. However, in this experiment, EFN secretion was quantified individually for each leaf, including both the "local" (i.e., treated) as well as the "systemic" (i.e., non-treated) leaves of the same branch.

In order to quantify CWIN activity, an independent set of eight plants from each species were selected and treated as described above. The nectary tissue was collected 1 h after JA-treatment and stored immediately on dry ice in 1.5 mL Eppendorf tubes. Samples were transferred to a portable Deep Freezer (https://www.thomassci.com/Equipment/General-Purpose-Refrigeration/_/25L-Super-Low-Temperature-Portable-Deep-Freezer?=&q=Ultra+Low+Freezer) and stored at −40°C until

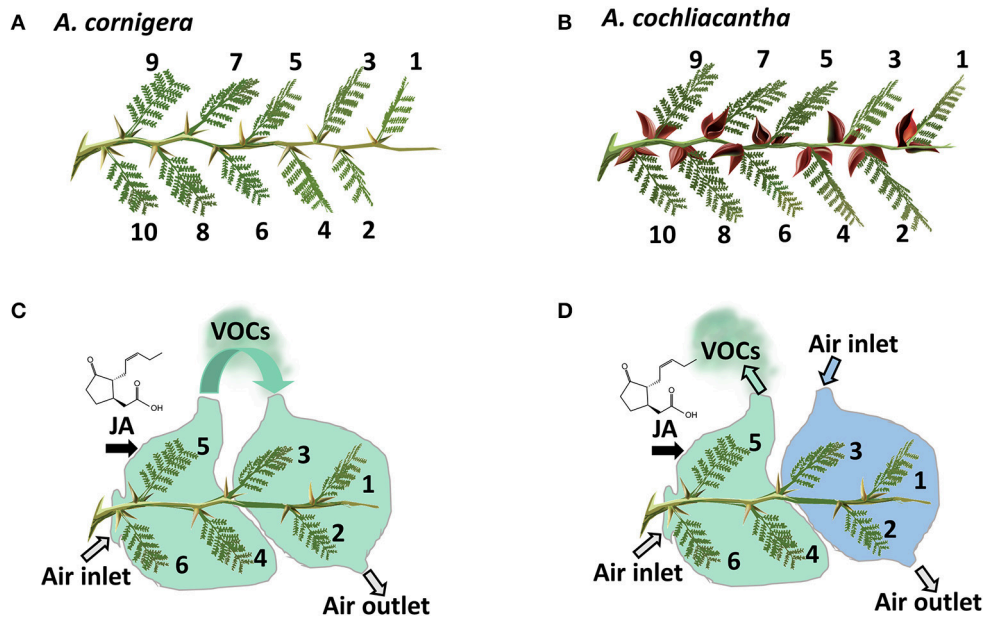

FIGURE 3 | Principles of experimental design. Of both study species (**A**, obligate ant-plant: *A. cornigera*; **B**, facultative ant-plant: *A. cochliacantha*), branches used in the experiments possessed at least 10 (last experiment: six) healthy leaves. Leaves were numbered in the order of their insertion on the branch, starting with the youngest fully expanded leaf. In the experiments aimed at understanding the role of volatile organic compounds (VOCs) as systemic signals, exposure of the youngest leaves (1-3) to the VOC-containing headspace of jasmonic acid (JA)-treated mature leaves (4-6) was controlled by bagging the treated leaves in PET foil and moving the airflow toward the younger leaves **(C)** or away from the plant **(D)**. See materials and methods section for details.

use. The activity of CWIN was quantified according to Millán-Cañongo et al. (2014) and Ruhlmann et al. (2010) with some modifications. Briefly, 0.10 g of frozen tissue was ground and mixed with 500 µL cold 50 mM HEPES/NaOH (pH 8.0, containing 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$ and 1 mM $CaCl_2$). Samples were incubated on ice for 10 min and then centrifuged at 13,000 g for 10 min at 4°C. The pellets that contained the cell walls with associated invertases were washed three times with 500 µL extraction buffer by resuspension and centrifugation as described above, each time discarding the supernatant. Then, the pellets were washed three times with 500 µL of 80 mM sodium acetate (pH 4.8). Then, 300 µL of 80 mM sodium acetate (pH 4.8) were added to the pellets, suspended, and the mixture was incubated at 37°C. Every 5 min, an aliquot of 20 µL was taken and mixed with 200 µL of hexokinase (HK) reaction solution ("glucose (HK) assay kit"; Sigma-Aldrich, http://www.sigmaaldrich.com). After reaching steady state, 100 µL of an aqueous 100 mM solution of sucrose was added, and the absorption was measured at 340 nm in a lQuant® Spectrophotometer, ThermoSpectronic, microplate reader every 5 min for 40 min with Gen 5 software (Biotek https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/).

Effect of VOCs on EFN Secretion

In order to investigate a putative role of VOCs on EFN secretion, we adapted the experimental design from (Heil and Silva- Bueno, 2007) in which the youngest leaves of a branch served as local "receiver" leaves to which air flow from the headspace of other leaves on the same branch was experimentally manipulated (**Figure 3**). In short, four plants each of *A. cornigera* and *A. cochliacantha* were selected as described above. Five branches that possessed at least six healthy leaves (of which the youngest leaves 1-3 served as receivers) were selected on each plant and were deprived of ants as described above. Then, the branches were subjected to one of the following treatments (see **Figure 3** for details). In the first treatment (**Figure 3C**), three mature leaves (4-6) were treated by spraying a 1 mM aqueous JA solution until the surfaces of all leaves were covered, allowed to dry, and then bagged in plastic foil ("Bratschlauch", Toppits, Minden, Germany; a PET foil that does not emit detectable amounts of volatiles). One side of the bag was left open and an open-flow system was created by placing a plastic tube (30 × 2 cm; inner surface lined with Bratschlauch) on the opposite side, creating a continuous air flow placing a ventilator (video card cooler "Evercool EC-4010," Steren, Mexico City, Mexico, supplied with 4.5 V) at the upper end of the tube. Then, air flow from the treated leaves was directed toward the three youngest leaves (1-3) on the same branch. In the second treatment (**Figure 3**, Panel D), the air flow from the treated leaves was directed away from the branch, leaving leaves 1-3 exposed to ambient air. As a control, we manipulated air flow was as in the first treatment, but leaves 4-6 were treated with water as a control. Ultimately, two branches of the same plant were exposed to air coming either from a JA-treated or a Milli-Q® water-treated branch of the other study plant species (i.e., leaves of *A. cornigera* were exposed to air coming from *A. cochliacantha* or *vice-versa*).

Collection and Analysis of VOCs

In order to compare the VOC profiles of *A. cornigera* and *A. cochliacantha* plants, two branches of each ten plants per species were selected and the youngest 10 leaves on one branch per plant were spray-treated with 1 mM of aqueous JA solution as described above, whereas the youngest ten leaves on the other branch were spray-treated with water as a control. After allowing leaves to dry, the branches were bagged in Bratschlauch. VOCs were collected over 24 h in a closed-loop system as described in Donath and Boland (1995), using micro-pumps (model DC 06/21 FK, Fürgut, Tannheim, Germany) and filters (1.5 mg of charcoal, CLSA- Filters, Le Ruissaeu de Montbrun, France). The VOCs were desorbed from the filters using 40 μL of dichloromethane with 1- bromodecane (98%, Aldrich) at a concentration of 100 ng μL^{-1} as an internal standard, and samples were injected directly into a gas chromatograph-electron impact ionization mass spectrometer (GC- EIMS) system (Agilent 7890 series gas chromatograph interfaced to an Agilent 5975 electron impact ionization mass-selective triple axis detector; Agilent Technologies Santa Clara, CA, USA). The separation was performed using a HP5- FAPP column (30 m long, 0.32 mm internal diameter and 0.5 mm film thickness) under the following conditions. Injector temperature 180°C, detector temperature 230°C, initial temperature 70°C, then ramped up at 5°C min^{-1} to 120°C, then ramped at 8°C min^{-1} to a final temperature of 210°C, which was maintained for 12 min. The mass spectra were analyzed with MassHunter 2017 by Agilent Technologies®, and compounds were preliminarily annotated with "NIST MS Search Program v.2.0g," Library version 11, and AMDIS version 2.71 from Agilent Technologies®, and then confirmed with authentic standards purchased from Sigma-Aldrich and Fluka Chemie (now Merck, purchased via Sigma-Aldrich, Toluca, Mexico).

Statistical Analysis

The all data obtained were subjected to *t*-student tests or ANOVA with posthoc Tukey-HSD. For the statistical analyses we used the program R® version 3.3.0 (R studio).

RESULTS

Effects of JA on EFN Secretion and CWIN Activity

Plants of *A. cornigera* secreted significantly more EFN on ant-inhabited and JA-treated ant-free branches as compared to water-treated ant-free branches ($p < 0.001$ for the comparisons ants *vs.* ant-free and JA *vs.* ant-free; $p > 0.05$ for ants *vs.* JA; see **Figure 4A**). In contrast, in the case of *A. cochliacantha*, only JA treatment had a significant effect on EFN secretion ($p < 0.001$; see **Figure 4B**), whereas no significant difference could be detected between ant-free branches and branches to which ants had access ($p > 0.05$, see **Figure 4B**). The differences between both species became even more pronounced when we investigated the systemic effects of local JA application (**Figure 5**). In the case of *A. cornigera*, application of JA to the nectaries on leaves 1, 5, and 10 significantly induced EFN secretion in the directly treated nectaries (difference between treated and control leaves: $p < 0.001$ at leaf positions 1, 5, and 10), whereas the EFN secretion rates on the systemic leaves of the treated branches showed no significant difference to the secretion rates on the corresponding leaves on untreated branches ($p > 0.05$ at leaf positions 2, 3, 4, 6, 7, 8, and 9; see **Figure 5A**). In contrast, all leaves on the treated branches of *A. cochliacantha* responded with a significant increase in EFN secretion to JA application to the nectaries on leaves 1, 5, and 10, independently whether they were "local" (i.e., directly treated) or "systemic" leaves (difference between leaves on treated and control branches: $p < 0.001$ at all 10 leaf positions, see **Figure 5B**).

The same patterns were observed in CWIN activity. In *A. cornigera*, CWIN activity in nectary tissue responded significantly ($p < 0.001$) to the direct JA application to nectaries

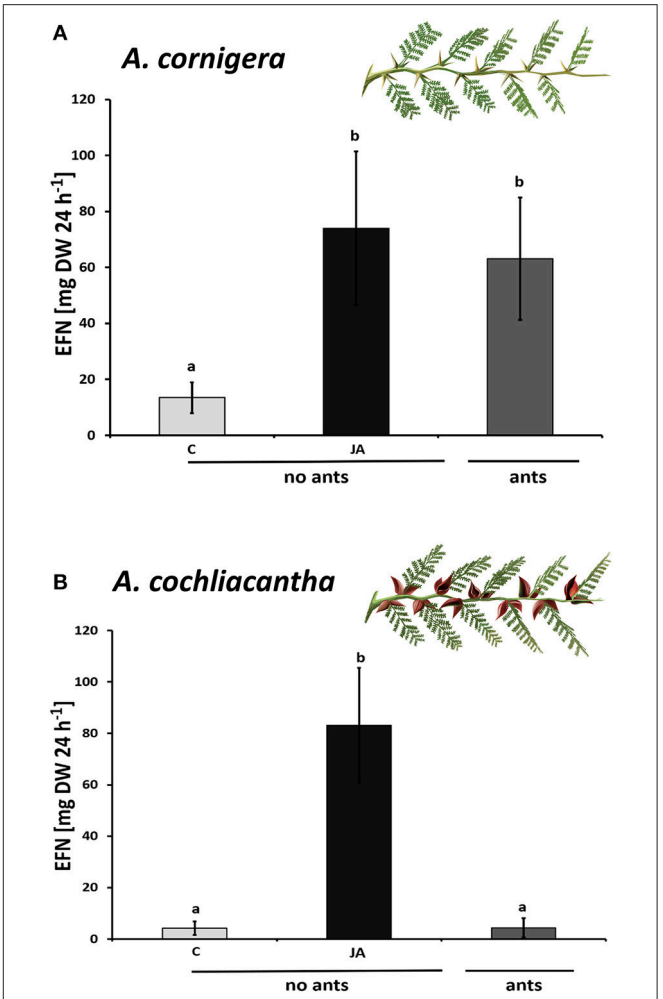

FIGURE 4 | Exogenous JA induces EFN secretion in *A. cornigera* and *A. cochliacantha*. Extrafloral nectar (EFN) secretion on ant-free branches of **(A)** *A. cornigera* and **(B)** *A. cochliacantha* responded to exogenous JA (black bars) as compared to controls (light gray bars). EFN secretion was also high on *A. cornigera* branches that were colonized by mutualistic (*P. ferrugineus*) ants but not on *A. cochliacantha* branches that were visited by generalist ants (dark gray bars). Bars represent means ± SD of EFN secretion rates in mg of sucrose equivalents per g of leaf dry mass and 24 h, different letters above bars indicate significant differences ($p < 0.001$, according to ANOVA followed by Tukey HSD, $n = 8$).

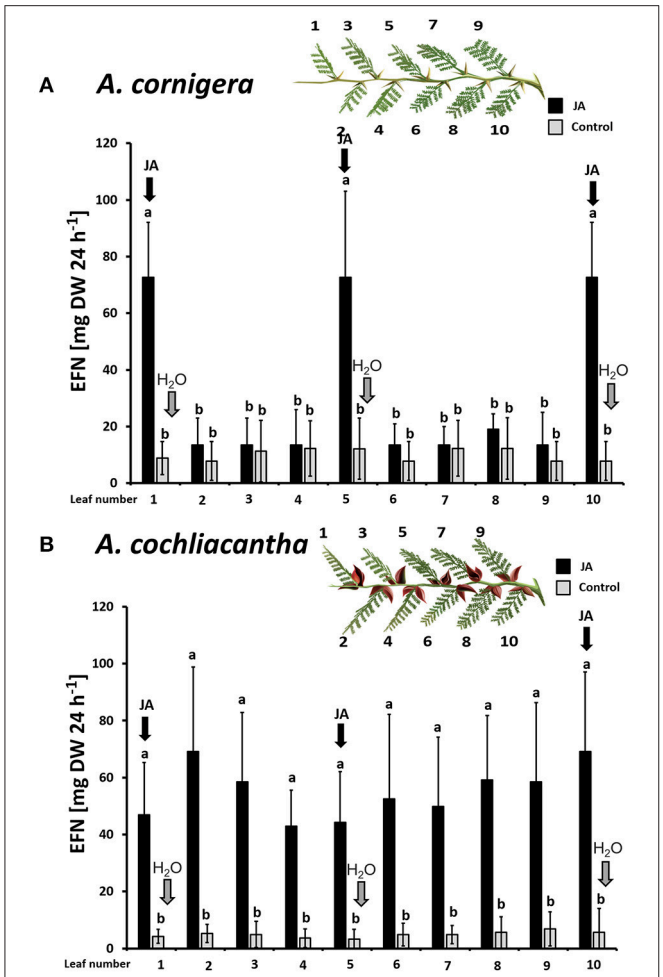

FIGURE 5 | Exogenous JA induces EFN secretion locally in *A. cornigera* but systemically in *A. cochliacantha*. Jasmonic acid (JA) was applied to leaves 1, 5, and 10 on ant-free branches of *A. cornigera* **(A)** or *A. cochliacantha* **(B)** and extrafloral nectar (EFN) secretion was quantified individually on all 10 leaves of these treated branches (black bars). Control branches (gray bars) received Milli-Q® water on leaves 1,5 and 10. Bars represent means ± SD of EFN secretion rates in mg of sucrose equivalents per g of leaf dry mass and 24 h different letters above bars indicate significant differences ($p < 0.001$, according to ANOVA followed by Tukey HSD, $n = 8$).

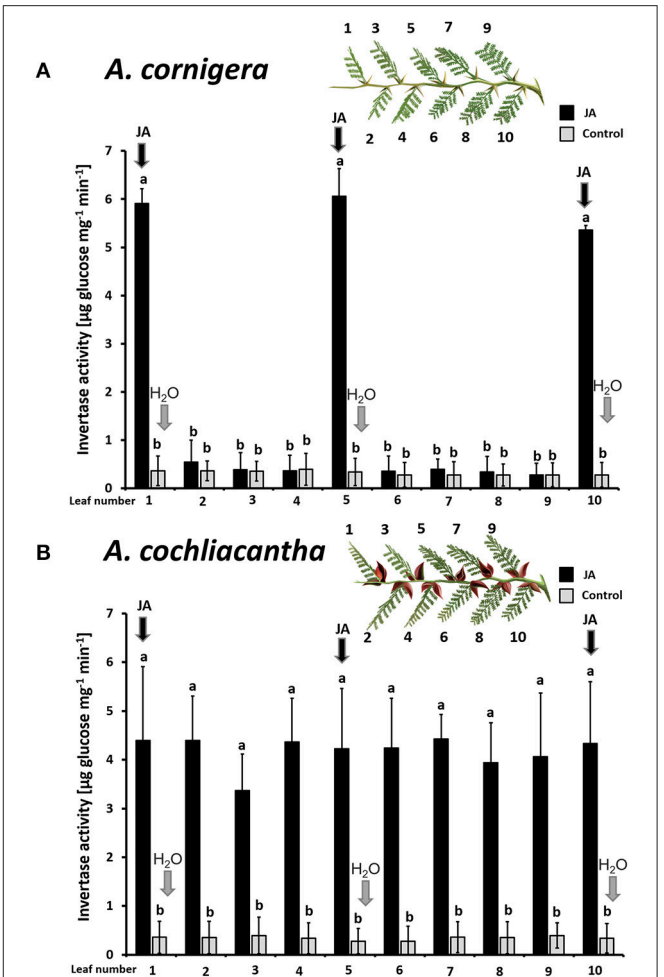

FIGURE 6 | Exogenous JA induces invertase activity in nectary tissue locally in *A. cornigera* but systemically in *A. cochliacantha*. Jasmonic acid (JA) was applied to leaves 1, 5, and 10 on ant-free branches of *A. cornigera* **(A)** or *A. cochliacantha* **(B)** and invertase activity was quantified individually on all 10 leaves of these treated branches (black bars). Control branches (gray bars) received Milli-Q® on leaves 1, 5, and 10. Bars represent means ± SD of invertase activity in µg of glucose per mg of nectary tissue per min, different letters above bars indicate significant differences ($p < 0.001$ according to ANOVA followed by Tukey HSD, $n = 8$).

on leaves 1, 5, and 10, but showed no significant differences between nectaries on leaves 2, 3, 4, 6, 7, 8, and 9 on JA-treated versus control branches ($p > 0.05$) (**Figure 6A**). In the case of *A. cochliacantha*, however, CWIN activity was significantly higher in all leaves on the treated branches as compared to the corresponding leaves on control branches ($p < 0.001$ at all 10 leaf positions), and no differences could be detected among the "local" and the "systemic" leaves on the treated branches (**Figure 6B**).

VOCs From *A. cochliacantha* Induce EFN Secretion in Both Species

When we treated mature leaves (4-6) of *A. cornigera* with JA to study the putative role of JA-responsive VOCs, the EFN secretion by these leaves was induced, as shown by the significant differences ($p < 0.001$) between EFN secretion rates observed on JA-treated leaves versus controls (**Figure 7A**). In contrast, the EFN secretion on the young leaves (1–3) of the same branches did not respond significantly to exogenous JA applied to the mature leaves, independently of whether the young leaves were exposed to the headspace of the treated, mature leaves ($p < 0.05$) or not (**Figure 7A,** treatments I and II). However, EFN secretion on young leaves (1–3) of *A. cornigera* was significantly induced after the exposure to the headspace of JA-treated leaves of *A. cochliacantha* ($p < 0.001$, see **Figure 7A**, treatment IVa). Correspondingly, EFN secretion on mature leaves of *A. cochliacantha* responded significantly to exogenous JA (**Figure 7B**), and the EFN secretion on young leaves was induced by the headspace of JA-treated mature leaves: that is, EFN secretion on leaves 1-3 was significantly

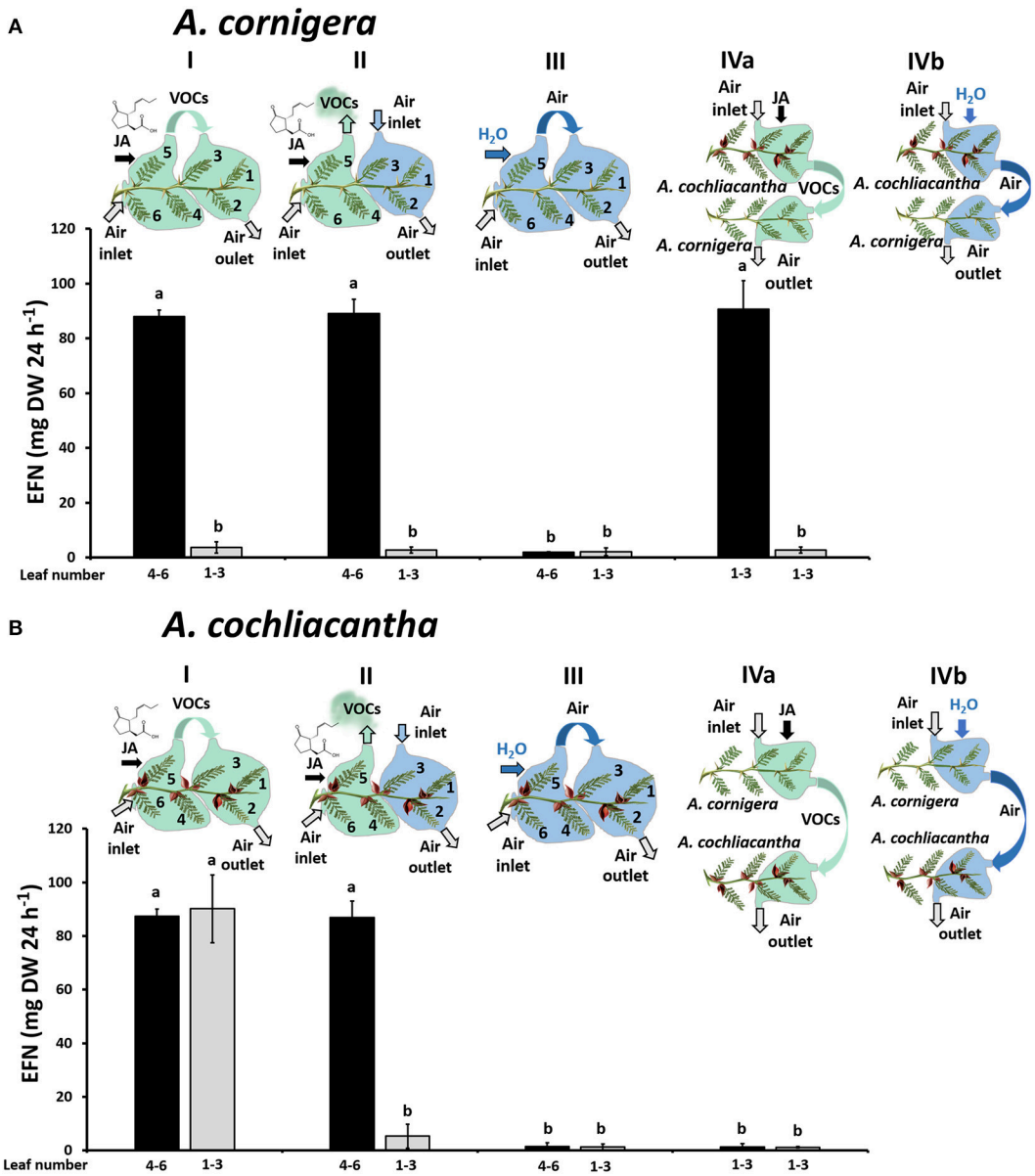

FIGURE 7 | The headspace of JA-treated *A. cochliacantha* branches induces extrafloral nectar (EFN) secretion in both plant species. The secretion of EFN on ant-free branches of *A. cornigera* **(A)** and *A. cochliacantha* **(B)** branches is depicted for young leaves (no. 1–3) and mature leaves (4–6) that were treated with JA or exposed to the headspace of differently treated leaves. Treatment I, mature leaves treated with JA, young leaves exposed to air from mature (i.e., induced) leaves. Treatment II, mature leaves treated with JA, young leaves exposed to environmental air. Treatment III, mature leaves treated with Milli-Q® water, young leaves exposed to air from mature (i.e., non-induced) leaves. Treatment IV, response of young leaves (1–3) to the headspace from heterospecific leaves (i.e., *A. cornigera* exposed to headspace of *A. cochliacantha* and vice-versa). The heterospecific emitter branches were either treated with JA (IVa) or with Milli-Q® water (control, IVb). Bars represent means ± SD of EFN secretion rates in μg per g leaf dry mass and 24 h, different letters above bars indicate significant differences ($p < 0.001$ according to ANOVA followed by Tukey HSD, $n = 4$).

higher ($p < 0.001$) on leaves that had been exposed to the headspace coming from JA-treated mature leaves (**Figure 7B**, treatment I) as compared to ambient air or the headspace of water-treated control leaves (**Figure 7B**, treatments II and III). Finally, no significant effect ($p > 0.05$) on EFN secretion by *A. cochliacantha* could be detected for the headspace of *A. cornigera*, independently of whether the *A. cornigera* branch had been treated with JA or not (**Figure 7B,** treatment IVa,b).

The Voc Blends of *A. cornigera* and *A. cochliacantha* Are Different

Both species responded to exogenous JA with the induced emission of various VOCs (**Table 1**). We could detect

TABLE 1 | Volatile organic compounds (VOCs) detected in the headspace of *A. cornigera* and *A. cochliacantha*.

			A. cornigera		*A. cochliacantha*	
Compound	**Peak**	**RT**	**JA**	**Control**	**JA**	**Control**
β-Pinene	1	7.18	6.0 ± 1.9	0.7 ± 0.4	6.7 ± 1.4	1.0 ± 09
(S)-(-)-Limonene	2	8.46	3.3 ± 2.1	2.1 ± 1.8	6.5 ± 3.1	ND
cis-β-Ocimene	3	13.15	9.3 ± 7.9	1.9 ± 1.2	18.8 ± 0.2	2.3 ± 0.5
β-Linalool	4	16.18	1.1 ± 1.0	ND	8.9 ± 0.9	1.9 ± 0.5
2,6-Dimethyl-1,3,5,7-octatetraene	5	17.29	2.9 ± 1.6	ND	8.6 ± 1.2	2.1 ± 0.2
α-Terpineol	6	18.32	1.5 ± 1.0	ND	7.6 ± 1.5	1.9 ± 0.5
cis-Hexenyl isovalerate	7	19.53	ND	ND	4.8 ± 0.1	ND
Longicyclene	8	21.27	ND	ND	1.1 ± 0.5	ND
α-Farnesene	9	23.28	ND	ND	5.1 ± 1.2	ND
α-Cubebene	10	24.64	ND	ND	3.7 ± 1.5	ND
Germacrene D	11	26.02	ND	ND	2.4 ± 1.1	ND
β-Caryophyllene	12	28.29	ND	ND	21.7 ± 3.2	ND

The headspace was sampled over 24 h after the treatment and compounds identified in ***Figure 8*** *as numbered peaks are listed according to their retention time (RT), values represent mean peak areas (×10^7) ± SD per g of dry mass of the emitting leaves of n = 10 independent samples per species and treatment, ND, Not detected. All compounds were confirmed by co-injection with commercial standards.*

six different compounds in the headspace of JA-treated *A. cornigera* branches, among which the monoterpene alcohols, α-terpineol and β-linalool, and 2,6-dimethyl-1,3,5,7-octatetraene ("dimethyl-octatetraene" in **Figure 8A**), were released only from JA-treated plants (**Figure 8A**). All six compounds that we detected in the headspace of JA-treated *A. cornigera* branches could also be detected in the headspace of JA-treated *A. cochliacantha* branches. However, *A. cochliacantha* emitted six additional compounds: *cis*-hexenyl isovalerate, the monoterpene, α-cubebene, and the sesquiterpenes longicyclene, germacrene, β-caryophyllene and α-farnesene (**Figure 8B**). According to t-tests performed separately for each VOC, JA treatment induced the emission of four out of six compounds significantly ($p < 0.005$) in case of *A. cornigera* and of all 12 compounds in case of *A. cochliacantha* (**Table 1**, **Figures 8A,B**).

DISCUSSION

In the present study, we show that EFN secretion by the obligate ant-plant, *A. cornigera,* responded in a modular manner to local JA-treatment, whereas in the facultative ant-plant, *A. cochliacantha*, EFN secretion responded more systemically. The activity of CWIN in the tissue of the individual nectaries closely resembled the patterns seen in EFN secretion rates in both species, confirming earlier reports that EFN secretion is controlled at the level of individual nectaries (Orona-Tamayo et al., 2013). In *A. cornigera*, an enhanced EFN secretion also correlated with the colonization by resident mutualistic *P. ferrugineus* ants, whereas generalist ants visiting the nectaries of *A. cochliacantha* did not exert any detectable EFN-inducing effect. A modular sanctioning of non-reciprocating symbionts has been reported from different types of mutualisms. However, the signals that allow for different spatial patterns in the production of rewards remain to be identified. Thus, the observation of different spatial patterns in the reward production by two closely related species made our system highly suitable to search for the causal mechanism that controls these patterns.

EFN Secretion in Both Species Responds to *A. cochliacantha* VOCs

Interestingly, the headspace of JA-treated leaves of *A. cochliacantha* induced EFN secretion in the systemic leaves of the same branch. As reported earlier, inducible VOCs can act as airborne "external" signals that control systemic responses to local damage (Heil and Ton, 2008; Scala et al., 2013; Loreto et al., 2014). In contrast, EFN secretion by systemic leaves of *A. cornigera* did not respond to the headspace of the JA-treated leaves of the same branch. This observation shows that in *A. cornigera,* VOCs do not serve as a systemic, EFN-inducing signal, and raised the question whether the two species studied here differ in the emission of VOCs, or in the responsiveness of EFN secretion to these VOCs. Indeed, the headspace of JA-induced *A. cochliacantha* branches readily induced EFN secretion in *A. cornigera*. Although an exposure of *A. cornigera* leaves to VOCs from *A. cochliacantha* is not likely to resemble a natural situation of ecological relevance, this finding clearly demonstrates that EFN secretion by *A. cornigera*, in principle, can be induced by exogenous volatile signals.

VOCs as Plant-to-Ant Signals

Which ones among the VOCs that were emitted from *A. cochliacantha* were responsible for the EFN-inducing effect? The role of VOCs in the signaling from host plants to their ants has been studied in various ant-plant systems. In *Acacia* spp. and *Macaranga* spp., VOCs emitted from facultative and obligate ant-plants were compared to identify compounds that

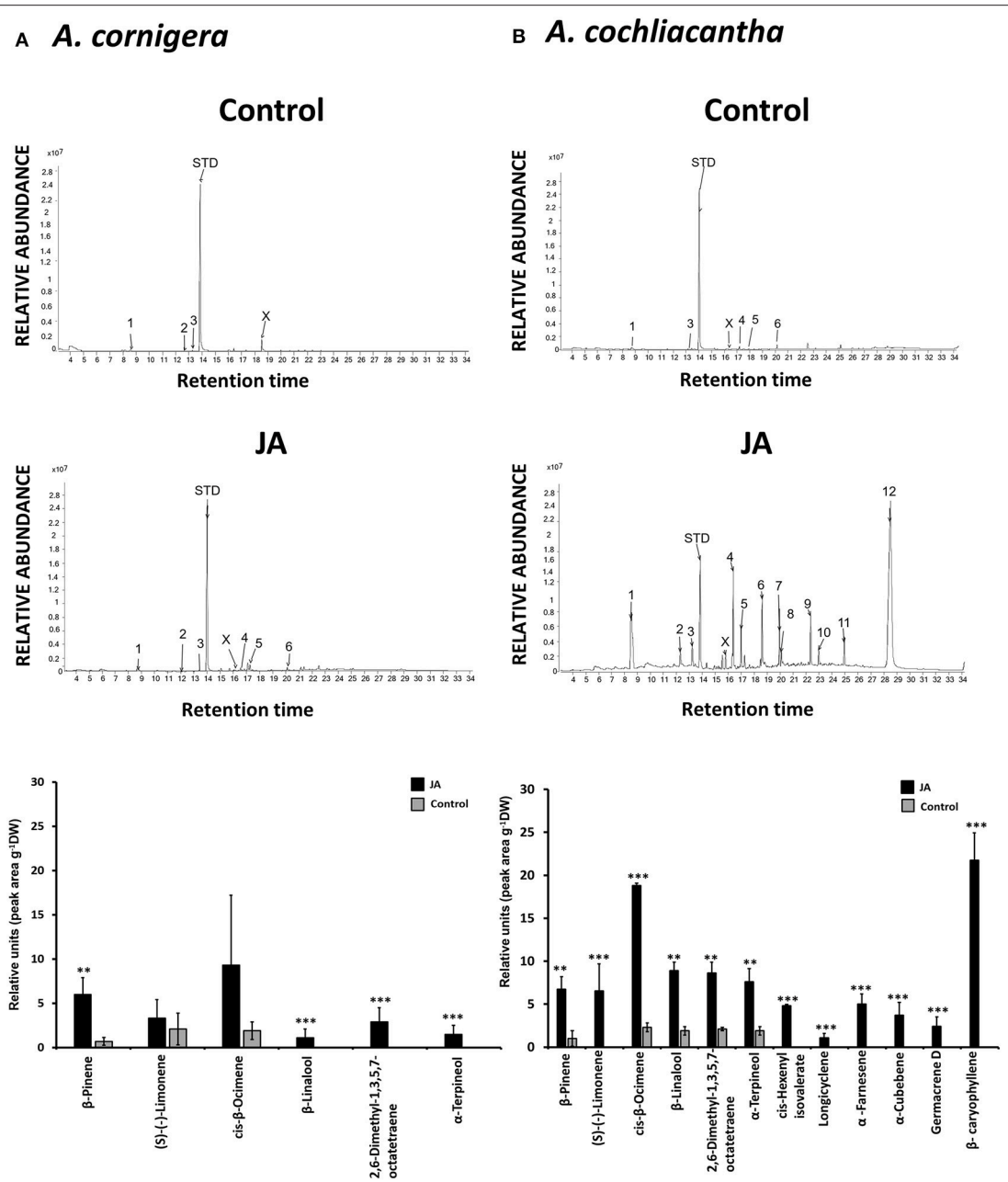

FIGURE 8 | Volatile organic compounds (VOCs) in the headspace of both Acacia species. Representative GC-spectra of VOCs are depicted for **(A)** *A. cornigera* and **(B)** *A. cochliacantha* branches treated with Milli-Q® water (control) or jasmonic acid (JA). Bar charts present average peak areas (×107) per g of dry leaf mass for $n = 10$ independent samples per species and treatment. Asterisks indicate the results of *t*-tests comparing control vs. JA-treatment (***$P < 0.001$, **$P < 0.005$).

might serve as host-finding cues for foundresses (Jurgens et al., 2006; Razo-Belman et al., 2018). Agrawal (1998) was the first to focus on the defensive aspect and studied plant-ants as a VOC-responsive mechanism of protection. Inui and Itioka (2007) found that diverse species of *Macaranga* myrmecophytes emitted different VOC profiles when damaged and that the aggressiveness of resident *Crematogaster* ants toward damaged leaves also differed among the host species. Using leaf pieces from different *Piper* species, Mayer et al. (2008) found that the resident ants responded more strongly to damaged leaves from obligate than from facultative *Piper* ant-plants, whereas Schatz et al. (2009) used the same approach to show that obligate plant-ants responded to leaf pieces of their host plant more strongly than a non-defending exploiter ant (Schatz et al., 2009). Curiously, this behavior could be elicited using, among other VOCs, pure hexanal (Schatz et al., 2009), a green leaf volatile already reported by Agrawal (1998) to elicit practically the same response as plant sap in *Azteka* plant-ants. That is, ubiquitous

plant VOCs that are emitted from most plants upon damage, or even infection (Heil, 2014; Quintana-Rodriguez et al., 2015, 2018), might be the triggers that ants use to detect host plant damage. More importantly, all the beforementioned six studies focused on the direct chemical communication from the plant to the ants, whereas in the present study, we focused on the VOC-mediated signaling within the plant.

Plant VOCs as Defence-Inducing Hormones

The headspace of *A. cornigera* caused no detectable induction of EFN secretion, which makes it reasonable to assume that the active VOCs can be found under those compounds that were emitted only, or in much higher amounts, by *A. cochliacantha*. Defence-inducing effects are commonly reported for green leaf volatiles such as (*E*)-2-hexenal or (Z)-3-hexenyl acetate (Scala et al., 2013; von Mérey et al., 2013; Loreto et al., 2014; Sharma et al., 2017). For example, *cis*-hexenyl isovalerate has been reported to induce EFN secretion in *Phaseolus lunatus* (Heil et al., 2008) and indeed, this compound could be detected only in the headspace of JA-treated *A. cochliacantha* branches. We could not detect other green leaf volatiles in our analyses, which might be partly due to the particular difficulties to collect small, highly volatile compounds under field conditions or to the detection threshold of our GC-MS analyses. Nevertheless, our control samples were practically free of detectable VOCs and all six VOCs that we detected in the headspace of *A. cornigera* had already been reported from this plant species (Razo-Belman et al., 2018). These facts make us confident that our results adequately resemble the major VOCs that are emitted from our study species. Five of the six compounds that were exclusive for *A. cochliacantha* were mono- or sesquiterpenes, a group of VOCs for which defense-inducing effects are much less frequently reported than for green leaf volatiles (Sharma et al., 2017). However, an artificial blend consisting of *R* - (–)-linalool, β-caryophyllene, methyl salicylate, *cis*-jasmone, (*cis*)-3-hexenyl acetate, β-ocimene, (3*E*)-4,8-dimethylnona-1,3,7-triene (DMNT) and (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) induced EFN secretion in lima bean (*Phaseolus lunatus*) (Kost and Heil, 2006), DMNT and TMTT induced pathogenesis-related (PR) genes and lipoxygenase (a central step in the synthesis of JA) in *P. lunatus*, and β-ocimene induced PR genes in the same species (Arimura et al., 2000). Likewise, a mixture of α- and β-pinene induced PR1 gene expression in *Arabidopsis* (Riedlmeier et al., 2017), whereas β-caryophyllene triggered membrane depolarization, which is a very early step in plant defense induction, in *Solanum lycopersicon* (Zebelo et al., 2012). Among these compounds, β-caryophyllene and β-ocimene were the quantitatively dominant compounds in the headspace of JA-treated *A. cochliacantha* branches, and β-caryophyllene was exclusive to this species. Taken together, our results make it highly likely that VOCs that are emitted only – or in much higher amounts–from *A. cochliacantha* leaves function as a systemic EFN-inducing signal, and that the strictly modular response in EFN secretion that we observed in *A cornigera* is caused by a reduced emission of these volatile signals, rather than a reduced responsiveness to the signals.

Optimized Rewarding by Modular vs. Systemic Responses

Modular responses in plant defense have been suggested to be driven by herbivores in order to optimize host sharing (Lee et al., 2017). However, in the case of our study system, it appears more likely that local EFN secretion enables *A. cornigera* to focus reward production on specific parts of the plant surface. EFN is a costly reward (Escalante-Pérez and Heil, 2012) and can be a limiting factor for ant colony growth (Byk and Del-Claro, 2011). In the case of Swollen-thorn Acacias, higher EFN secretion rates can shift the competitive balance between defending mutualist ants and non-defending exploiters to the benefit of the mutualists (Heil, 2013). The reduced emission of VOCs by *A. cornigera* is likely to represent a consequence of the frequently proposed reduction of direct defense traits in obligate ant-plants (Janzen, 1966; Rehr et al., 1973; Koricheva and Romero, 2012), rather than a specific adaptation to avoid a systemic induction of EFN secretion.

We also hypothesized that systemic EFN secretion enables a facultative ant- plant to attract more ants and gain a better defensive service when it is required. Although being reasonable (Agrawal and Rutter, 1998), surprisingly little evidence has been reported to support this assumption. In fact, the defensive effects of EFN secretion are highly context-dependent (Heil, 2015; Jones et al., 2017) and EFN secretion can even be counterproductive if ants start to exclude other, more efficient defenders Koptur et al., 2015). Inducing EFN secretion with exogenous JA increased the number of defending ants and decreased the number of herbivores showing up on *Macaranga tanarius* plants (Heil et al., 2001), and similar patterns were found on *P. lunatus* tendrils that were exposed to VOCs or treated with JA to enhance EFN secretion (Kost and Heil, 2008). However, all leaves had been treated in these studies, making a separation of local and systemic effects impossible. A study at the ecosystem level showed that ant abundance increased with higher EFN secretion rates and presented evidence for a strong competition among the ants for this valuable food reward (Lange et al., 2017). That ants compete for EFN has been reported from different systems (Blüthgen and Fiedler, 2004; Xu and Chen, 2010; Lange et al., 2017), which makes it likely that, under most circumstances, enhanced amounts of EFN that are secreted on larger areas of a facultative ant- plant should enhance the number of attracted ants (**Figure 1**).

CONCLUSIONS

In the case of an obligate ant-plant, a modular rewarding of resident ants (**Figure 2**, and Supplementary Video 1) should allow for an optimized investment in protection, because these ants defend only a restricted part of the plant. In contrast, the protection of a facultative ant-plant should increase when higher numbers of visiting ants are recruited via an enhanced investment in reward production. Interestingly, volatile signals represent a molecular mechanism that controls systemic responses to local events and that can generate marked differences in the provisioning of rewards among closely related plant species that engage in different types of food-for-protection mutualisms.

AUTHOR CONTRIBUTIONS

OH-Z and RR-B designed and performed the field experiments, analyzed the data and wrote a first version of the manuscript. MH helped with the design of the experiments and data analyses and all authors worked on the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01076/full#supplementary-material

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Ant-Pollinator Conflict Results in Pollinator Deterrence but no Nectar Trade-Offs

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Direct and indirect negative interactions between ant guards and pollinators on ant-plants are expected for two reasons. First, aggressive ants may deter pollinators directly. Second, pollinators benefit from plant investment in reproduction whilst ants benefit from plant investment in indirect defense, and resource allocation trade-offs between these functions could lead to indirect conflict. We explored the potential for ant-pollinator conflict in a Mexican myrmecophile, *Turnera velutina,* which rewards ants with extrafloral nectar and pollinators with floral nectar. We characterized the daily timing of ant and pollinator activity on the plant and used experiments to test for direct and indirect conflict between these two groups of mutualists. We tested for direct conflict by quantifying pollinator responses to flowers containing dead specimens of aggressive ant species, relative to unoccupied control flowers. We assessed indirect conflict by testing for the existence of a trade-off in sugar allocation between ant and pollinator rewards, evidenced by an increase in floral nectar secretion when extrafloral nectar secretion was prevented. Secretion of floral and extrafloral nectar, activity of ants and pollinators, and pollen deposition all overlapped in daily time and peaked within the first 2 h after flowers opened. We found evidence of direct conflict, in that presence of ants inside the flowers altered pollinator behavior and reduced visit duration, although visit frequency was unchanged. We found no evidence for indirect conflict, with no significant difference in the volume or sugar content of floral nectar between control plants and those in which extrafloral nectar secretion was prevented. The presence of ants in flowers alters pollinator behavior in ways that are likely to affect pollination dynamics, though there is no apparent trade-off between plant investment in nectar rewards for pollinators and ant guards. Further studies are required to quantify the effect of the natural abundance of ants in flowers on pollinator behavior, and any associated impacts on plant reproductive success.

Keywords: ***Turnera velutina,*** **ant-plant, floral nectar, extrafloral nectar, resource allocation, indirect interactions, Myrmecophily**

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INTRODUCTION

Extrafloral nectaries, domatia, and food bodies are all means by which ant-plants (comprising myrmecophiles and myrmecophytes) (Rosumek et al., 2009; Del-Claro et al., 2016) attract and support ants by providing nesting sites or nutrients (Rico-Gray and Oliveira, 2007; Rosumek et al., 2009). In return, ants attack herbivores, prune climbing vines and prevent fungal and microbial

infestation on plant tissues (Bentley, 1977; Rosumek et al., 2009). This mutualistic interaction is termed myrmecophily.

Interactions involving two or more types of mutualists of a single host are common in nature, but multispecies interactions are much less studied than pairwise and intraguild mutualisms (Strauss, 1997; Tscharntke and Hawkins, 2002; Strauss and Irwin, 2004; Adler, 2008; Melián et al., 2009; Koptur et al., 2015). To date, most research on plant-animal interactions has focused on pairwise relationships (e.g., plant-herbivore, plant-pollinator, plant-fungus) in isolation from the community in which they are embedded (Strauss, 1997; Herrera, 2000; Dáttilo et al., 2016; Del-Claro et al., 2018). This pairwise approach necessarily oversimplifies reality (Herrera, 2000) since plants interact sequentially or simultaneously with each of pollinators, herbivores, herbivore predators and pathogens (Armbruster, 1997). Furthermore, plant interactions with one partner or guild can also affect relationships with other groups or guilds (Armbruster, 1997) and alter outcomes from mutualistic to antagonistic (Strauss, 1997; Strauss et al., 1999; Herrera, 2000; Strauss and Irwin, 2004; Del-Claro et al., 2016). As a result, a growing number of studies are focusing on multispecies and multitrophic interactions (Melián et al., 2009; Fontaine et al., 2011; Nahas et al., 2012; Pineda et al., 2013; Dáttilo et al., 2016). It might be expected, for example, that the presence of predatory ants can influence pollinators, with top-down effects on plant fitness. This makes ant-plants, which rely on ants for defense against herbivores and on pollinators for seed set, a model tritrophic system in which to explore the dynamics of multispecies and multitrophic interactions.

Here, we focus on disentangling ant-pollinator interactions that occur when both mutualists share a host plant. Previous work has revealed evidence of ant-pollinator conflict in such systems (Yu and Pierce, 1998; Stanton et al., 1999; Gaume et al., 2005; Ness, 2006; Palmer and Brody, 2007; Frederickson, 2009; Stanton and Palmer, 2011; Malé et al., 2012; Assunção et al., 2014; LeVan et al., 2014; Hanna et al., 2015). Ant-pollinator conflict is expected for two main reasons. Firstly, both mutualists share with their host interest in different plant functions. Pollinators benefit from plant resource allocation to reproduction (i.e., flowers, floral nectar (FN) and pollen), whilst ants benefit from allocation to growth and defense [i.e., vegetative structures bearing extrafloral nectar (EFN) or domatia] (Yu and Pierce, 1998; Frederickson, 2009; Palmer et al., 2010). This could result in a conflict mediated by plant rewards, known as indirect conflict. Floral and extrafloral nectar share sugar as a common currency, providing potential for a trade-off and also a means of quantifying investment in each. Secondly, because ant guards actively defend their host plant as a means of protecting food and/or nesting sites, they may also repel or attack pollinators (Ness, 2006; Stanton and Palmer, 2011; Chamberlain and Rudgers, 2012), and this drawback of ant guards is known as direct conflict.

Castration is an extreme example of direct ant-pollinator conflict in which guarding ants destroy or consume the reproductive meristems, floral buds or flowers of their host plant (Yu and Pierce, 1998; Stanton et al., 1999; Gaume et al., 2005; Palmer and Brody, 2007; Frederickson, 2009; Malé et al., 2012). Such castrating behavior inevitably reduces availability of flowers and hence floral rewards for pollinators. It has been suggested that the ultimate cause of castration by patrolling ants is promoting reallocation of plant resources from reproduction to growth (Yu and Pierce, 1998; Frederickson, 2009; Malé et al., 2012), and hence increases the availability of resources on which ant colonies depend. In ant species that are obligate inhabitants of ant-plants, colony size is limited by the number of domatia (Fonseca, 1993, 1999; Orivel et al., 2011), which is positively correlated with plant investment in growth. Consequently, resource allocation strategies toward these two mutualists should be approached in a linked way because plant investment toward growth may come at the cost of investment to reproduction, and vice versa. And so, plant investment in rewards for each mutualist reward may be affected, either positively via linkage, or negatively via trade-offs. Furthermore, because plants interact with both mutualists simultaneously the presence of one mutualist may increase or decrease presence of the other.

Even in those species that do not castrate their host, ants' aggressive behaviors might threaten and deter pollinators, compromising plant reproduction (Ness, 2006; Assunção et al., 2014; LeVan et al., 2014). Avoidance of such direct conflict has been suggested to explain plant architecture or behaviors that reduce spatial (Raine et al., 2002; Malé et al., 2015; Martínez-Bauer et al., 2015) or temporal overlap of ant guards and pollinators (Gaume and Mckey, 1999; Gaume et al., 2005; Nicklen and Wagner, 2006; Ohm and Miller, 2014; Malé et al., 2015), and the presence of ant-repelling compounds which exclude ants from flowers when pollen is released (Junker et al., 2007; Willmer et al., 2009; Ballantyne and Willmer, 2012).

Extrafloral nectar is a key resource mediating multispecies interactions in many plant communities, and plants bearing extrafloral nectaries comprise up to a third of species in some biomes (Dyer and Phyllis, 2002; Rudgers and Gardener, 2004; Davidson and Cook, 2008; Dyer, 2008), particularly in tropical dry forests, savannas and *cerrados* (Rico-Gray and Oliveira, 2007; Assunção et al., 2014). The importance of ant-plants as food resources for mutualists in a given plant community is enhanced if these plants also secrete FN and pollen for pollinators. Management of ant-pollinator conflict in such a way that the crucial services provided by both mutualist groups are maintained is thus likely to be part of the adaptive landscape of many plant species. Ant-plants with extrafloral and floral nectaries represent an excellent system in which to test for trade-offs in resource allocation, competition amongst mutualistic guilds, and assess whether plant strategies minimize direct and indirect conflicts between their mutualists. To our knowledge, no study has addressed both direct and indirect ant-pollinator conflict in a single ant-plant system.

Here we tested for direct and indirect ant-pollinator conflict on a Mexican endemic ant-plant, *Turnera velutina*. In particular, we assessed whether *Turnera velutina* reduces the potential for conflict through the daily timing of FN and EFN release. We also tested for potential indirect (nectar-mediated) and direct (deterrence) conflicts between ants and pollinators. We

addressed the following specific questions: (i) What are the daily timings of nectar reward secretion, ant activity, and floral visitation? (ii) Does the presence of patrolling ants deter pollinators from the flowers? (iii) Do ant species vary in their deterrence for pollinators? (iv) Are *T. velutina* plants able to re-allocate extrafloral nectar resources into floral nectar resources (increasing reward availability to flower visitors)?

MATERIALS AND METHODS

Study Site and System

Field experiments were conducted in coastal sand dunes at the CICOLMA Field Station in La Mancha, Veracruz, in the Gulf of Mexico (19°36′ N, 96°22′W, elevation < 100 m). The climate is warm sub-humid, with a rainy season during the summer (June to September), an annual precipitation of 1,100–1,500 mm, and a mean annual temperature ranging between 24 and 26°C (Travieso-Bello and Campos, 2006). Experiments were carried out in November 2014 at four sites with high densities of *Turnera velutina* (Passifloraceae). Greenhouse experiments were conducted in a shade house at CICOLMA.

Turnera velutina is an endemic perennial shrub (Arbo, 2005) and myrmecophile (Cuautle and Rico-Gray, 2003). At La Mancha, *T. velutina* is patrolled by at least seven ant species (Cuautle et al., 2005; Zedillo-Avelleyra, 2017) and its main herbivores are the specialist caterpillars of the butterfly *Euptoieta hegesia* (Nymphalidae). Extrafloral nectar is provided in paired cup-shaped glands located at the bottom of the leaf blade at the junction with the leaf petiole, on the underside of the leaves (**Figure 1A**, Elias et al., 1975; Villamil et al., 2013). Although it flowers year-round, flowering peaks during summer (Cuautle et al., 2005). Flowers last one day, are animal-pollinated (Sosenski et al., 2016) and provide FN at the base of the corolla (**Figure 1**). Honeybees (*Apis mellifera*) are the dominant pollinators at La Mancha, accounting for 94% of the visits (Sosenski et al., 2016) and collect both pollen and floral nectar, but the role of other floral visitors is yet to be investigated as effective pollinators. There is no spatial segregation of patrolling ants and floral visitors in *Turnera velutina* since flower buds emerge from the axillary meristems of leaves bearing extrafloral nectaries (Villamil, 2017). Furthermore, EFN volume and sugar content are higher at the flower stage than for either buds or fruits (Villamil, 2017).

FIGURE 1 | Flower of Turnera velutina **(top)** with arrows indicating the location of the floral nectaries at the base of the corolla, between the petals, and leaves **(bottom)** with arrows indicating the location of extrafloral nectaries, on the underside of the leaves.

Fieldwork Methods

Mutualist Activity Curves: Patrolling Ants and Pollinators

We quantified daily activity of patrolling ants and flower visitors on *T. velutina* by surveying flowers ($n = 120$ plants, $n = 1{,}604$ flowers) and their associated leaves at all four La Mancha sites in November 2014. We observed all open flowers for 2 min every hour throughout anthesis (0800–1300 h), with one observer at each site. Every hour, we counted the total number of floral visitors and ants patrolling extrafloral nectaries across all flowers within a site. We sampled the same sites over multiple days. Since these are one-day flowers, we considered each site-day as a replicate ($n = 10$ site-days; 43.23 ± 2.89 flowers/site-day), and incorporated site-and-day effects into our statistical modeling (see below).

Nectar Secretion and Pollen Deposition Curve

Nectar secretion and pollen deposition data were collected from 4 sites within CICOLMA over 5 consecutive days in September 2015. We visited a single site per day (with one exception which was visited twice) and sampled FN and EFN secretion rate and pollen deposition from 1 flower per plant for 5 plants per site ($n = 20$ plants). Flowers were sampled every hour during the anthesis period (0800–1300 h). Flowers were bagged with tulle bags before anthesis and FN and EFN were collected every hour during anthesis. The first collection was taken as soon as the corolla was fully open. Flowers were re-bagged between measurements to avoid nectar consumption and a masking tape band with Tanglefoot was applied on the stem below the flower to exclude ants for the duration of the experiment. FN was extracted using a 1 μl capillary inserted into each of the five nectaries

in a single flower (Minicaps Disposable capillaries, Hirschmann Laborgerate, In 20°C, ISO 7550, R<0.5%, CV<1.0%, Germany). EFN was also collected from the glands using 1 μl capillaries. Nectar volume was measured using a digital caliper (Mitutoyo Digimatic) and sugar concentration was determined in °Brix (g sucrose per 100 g solution) using a 0–50°B hand held refractometer (Reichert, Munich, Germany). To obtain all of the sugar from extrafloral nectaries, the glands were washed with 2 μl of deionized water using a 0–5 μl micropipette and the sugar concentration of the wash was quantified using the refractometer. Total sugar content in FN and EFN was calculated from volume and °Brix values according to Comba et al. (1999) using the formula:

$$s = dvC/100$$

where s is the sugar content (μg), v is the nectar or wash volume (μl), and d is the density of a sucrose solution at a concentration C (g of sucrose per 100 g solution) as read on the refractometer. The density was obtained according to Comba et al. (1999) using the formula:

$$d = 0.0000178C^2 + 0.003791C + 0.9988603$$

A different flower from each of these twenty plants was chosen and marked, but never bagged. We collected one stigma every hour, to sample the pollen deposited 1 h, 2 h, and 3 h post-anthesis. A small proportion of flowers contained a fourth stigma, contributing to a small dataset for 4 h post anthesis. Stigmas were stored individually in labeled Eppendorf tubes. Stigmas were individually mounted on slides for fresh squash glycerin preparations. Slides were sealed using nail polish and kept in a fresh and dry environment at 22°C. Each slide was labeled with the site, day, hour, and plant identity. The number of pollen grains on each stigma was counted under a microscope, and changes in numbers at each time interval plotted to generate the pollen deposition curve.

Direct Conflict

To test whether non-ant flower visitors detect and avoid flowers with ants, visitation was observed in flowers with and without dead ants for 4 flowers on each of 40 plants ($n = 160$ flowers). Plants > 80 cm in height were haphazardly selected and flowers bagged at 0700 before anthesis. Once the corollas had opened, three ant corpses from a single ant species (either *Dorymyrmex bicolor*, *Brachymyrmex* sp., or *Paratrechina longicornis*) were placed inside each of three flowers/plant. A fourth flower was left as an ant-free control. Ant corpses were placed on the inner surface of the petals in each flower. These species were chosen because they were amongst the most abundant species (see Results), displayed patrolling behaviors on *T. velutina*, and were detected as potentially differing in their effects on *Apis mellifera* pollinators by a previous study (Villamil et al. unpublished data). Additional flowers were removed from the plant to standarise floral display across all individuals. Ants were killed in 50% ethanol, which was allowed to evaporate for 30 min at ambient temperature (28–35°C) from all samples to prevent ethanol vapors from influencing pollinator behavior. Flowers containing dead ants were observed for 20 min, recording pollinator identity, visit frequency and duration, and associated pollinator behaviors. Observations were conducted during October-November 2016 with four simultaneous observers collecting data from different plants.

When assessing the effects of ants inside the flowers on pollinator visitation we only considered visits by *Apis mellifera,* since this is the dominant *T. velutina* pollinator in this population (Sosenski et al., 2016) and accounted for 91% of the visits in this experiment. We classified honeybee behaviors as "inspection" or "contact." Inspection behaviors comprised either approaching or hovering over a particular flower without landing. Contact behaviors were those that occurred inside the flower, between landing and take-off, and comprised foraging on pollen or nectar resources or standing on the petals, anthers or stigmas.

Indirect Conflict

To test possible trade-offs in plant resources between FN and EFN we conducted a greenhouse experiment on 72 plants from 18 different maternal lines (2–4 siblings per maternal family, generated from field individuals). Plants were kept under greenhouse conditions in a shade house located within CICOLMA field station. Plants were grown in 1L plastic pots with a substrate of local soil and vermiculite (50:50) and watered every other day (for rearing details see: Ochoa-López et al., 2015). During the experiment, plants were watered every night, and extrafloral nectaries were sprayed with water to wash away any nectar secretion from previous days and to prevent fungal infections on the glands. Pre-anthesis buds were bagged either the night before or during the morning before anthesis to prevent any nectar theft by unexpected insects that may occasionally enter the shade house.

Pairs of maternal siblings were chosen and randomly assigned to either control or experimental groups. The extrafloral nectaries in all leaves of experimental plants were clogged by applying Mylin transparent textile paint in the nectary cup. Extrafloral nectaries from control plants were left intact (unclogged) and droplets of the same paint were applied on the leaf blade above the glands to match any unintended effects on plants across treatments. Very young floral buds were marked, and their extrafloral nectaries clogged from their emergence, throughout their development, and until anthesis. The droplets on the extrafloral nectaries or leaf blades were checked daily and replenished when required, especially when a new leaf emerged in order to guarantee uniform and continuous application of the clogging treatment across all leaves.

FN secretion was measured before the clogging treatment was applied, and once again after the young clogged/marked buds became flowers. The aim was to test whether flowers that were unable to secrete EFN invested more sugar resources in floral nectar. FN was collected from control and treatment flowers between 1300 and 1500 h using a 1 μl microcapillary pipette. Nectar volume and total sugar mass was estimated and calculated as described above.

Statistical Methods

All statistical analyses were conducted in R version 3.23 (R Core Team 2016). All mixed effects models were fitted using the 'multcomp' R package (Bates et al., 2016) and *post hoc* Tukey comparisons were fitted using the 'multcomp' R package (Hothorn et al., 2008), unless stated otherwise.

Mutualist Activity and Reward Secretion Curves

To test whether ant or pollinator activity changed over daily time, we fitted a Poisson mixed model with either the number of ants patrolling or the number of floral visitors as the response variable. We fitted time of day as a fixed effect, with linear and quadratic terms to detect non-linear activity patterns over time. The number of flowers per site was fitted as a log-transformed offset to control for floral display, since we recorded visitor counts per site rather than counts per individual flower (see fieldwork methods). Flowers of *T. velutina* last for a single day, and because multiple flowers were sampled on a given site on a given day, we fitted site identity as a random effect to account for differences between site and day variation in variables that could influence ant abundance, such as resource availability, ant diversity, or the abundance/proximity of ant nests. We also included an observation-level random effect (OLRE) where each data point receives a unique level of a random effect to control for overdispersion (Hinde, 1982).

To test if FN and EFN secretion changed over the anthesis period we fitted a Poisson mixed model independently for each nectar type, with sugar mass (μg) as the response variable. Nectar sugar content is usually estimated in μg, and we report our raw data in such units. However, to facilitate model convergence we re-scaled our response variable (sugar content) from μg to g, and rounded it to the next integer to better fit a Poisson distribution. We fitted time of day as a fixed effect, with linear and quadratic terms to detect non-linear activity patterns over time. We fitted plant identity as a random effect, and, included an observation-level random effect.

Timing of Daily Activity and Secretion Peaks

We computed the time at which mutualist activity and nectar secretion reached their maximum by calculating the time at which the slope (i.e., the differential of the fitted model with respect to time) for each variable is zero and then solving for hour, as follows:

$$
\begin{aligned}
y &= \beta_{hour} * hour + \beta_{hour^2} * hour^2 \\
\frac{dy}{d_{hour}} &= \beta_{hour} + 2\beta_{hour^2} * hour \\
0 &= \beta_{hour} + 2\beta_{hour^2} * hour \\
\frac{-\beta_{hour}}{2\beta_{hour^2}} &= hour \\
hour &= -\frac{1}{2} * \frac{\beta_{hour}}{\beta_{hour^2}}
\end{aligned}
$$

Direct Conflict

The effect of different ant species on the visitation frequency was tested using Poisson mixed effects models. We fitted the number of visitors as the response variable, and ant species inside the flower (Control, *Dorymyrmex bicolor*, *Brachymyrmex* sp., or *Paratrechina longicornis*) was fitted as a fixed effect. Because these are 1 day flowers, plant-day identity was chosen as a random effect to control for individual variation in floral and extrafloral nectar investment. We also included an observation-level random effect. *Post hoc* Tukey comparisons were used to test differences in visit duration between the four treatments.

We tested whether ant species inside the flower differed in their effect on the likelihood with which a pollinator displayed an inspection behavior using a binomial mixed model. The presence or absence of inspection behaviors was coded as the response variable and ant species was fitted as a fixed effect. As random effects we fitted the plant-day identity, and the visitor identity. For those visits where the pollinator displayed an inspection behavior, we fitted the proportion of time per visit spent displaying inspection behaviors using a Gaussian mixed model with logit transformation for data normality. Ant species was included as a fixed effect, and plant-day identity, and the visitor identity were fitted as random effects.

Finally, differences in the duration of inspection or contact behaviors in flowers containing different ant species were analyzed using Gamma mixed models. Mixed effects models were fitted independently for each behavior, but using the same model structure fitting visit duration per flower as the response variable. Ant species inside the flower was fitted as a fixed effect. Plant-day identity was chosen as a random effect to control for individual variation in floral and extrafloral investment, and daily weather variations. We also included an observation-level random effect. *Post hoc* Tukey comparisons were used to test differences in visit duration between the four treatments.

Indirect Conflict

For each plant, we estimated the difference in FN produced before and after the extrafloral nectaries were clogged as follows: $D_{FN} = Post_{FN} - Pre_{FN}$, where D-FN is the difference, $Post$FN is the FN production after the extrafloral nectaries secretion was prevented and PreFN is the FN production before the extrafloral nectaries were clogged. Differences in volume and sugar content between control and experimental flowers were tested using mixed effects models. Both variables had normal distributions and so Gaussian mixed effects models were fitted using the same model structure: The clogging treatment was fitted as a fixed effect, and the maternal family was fitted as a random effect to independently explain variation in both nectar volume and sugar content.

RESULTS

Mutualist Activity, Reward Secretion, and Pollen Deposition Curves

Activity curves show that both patrolling ants and floral visitors were most active within the first 2 h post-anthesis (**Figure 2**), although the visitation peak by potential pollinators predicted from model estimates was on average over an hour before the predicted peak for ant activity (9 min post-anthesis for potential pollinators, 90 min post-anthesis for ant patrolling; **Table 1**).

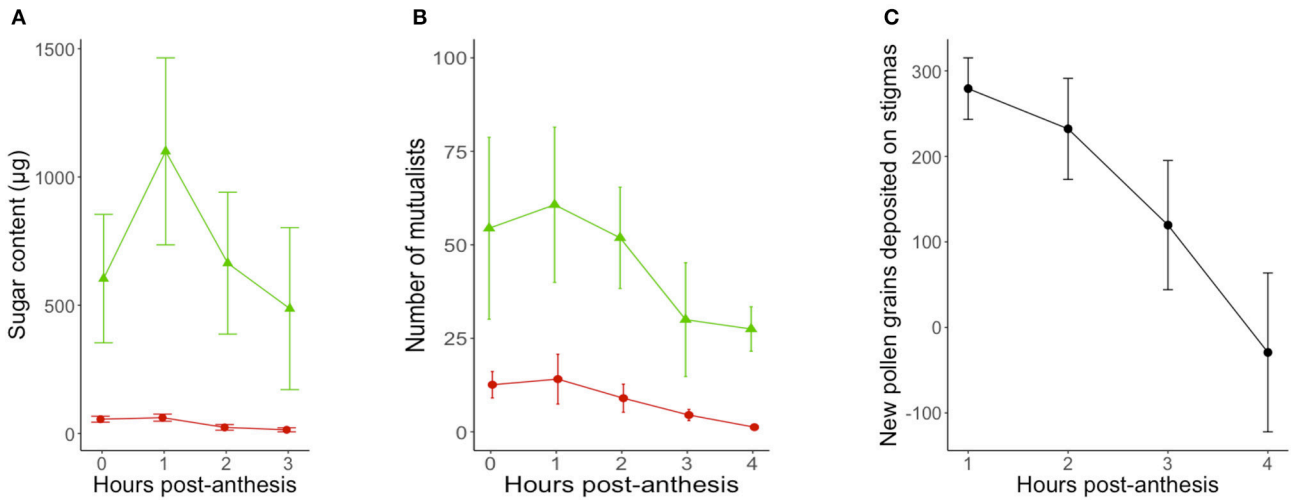

FIGURE 2 | Timing of **(A)** reward secretion ($n = 20$ flowers-leaves), **(B)** mutualist activity (n = 1,604 flowers; $n = 10$ sites/day) and **(C)** pollen deposition ($n = 20$ flowers) in *Turnera velutina* during the anthesis period (08:30–12:30), showing raw data from field observations (mean ± se). Red circles show floral nectar and the activity of pollinators in flowers, whilst green triangles show extrafloral nectar and the activity of ants at extrafloral nectaries.

TABLE 1 | Model statistics for the timing of mutualists activity, reward secretion and pollen deposition in *Turnera velutina*.

	Response	Fixed effects	N	Estimate	LRT	*P*-value		Random effects	Variance	SD	Maxima estimates		
											Peak hour	mpa	Time of day
Mutualist activity	Patrolling ants (number)	Log(flowers)	33	0.804	7.967	0.004	***	OLRE Site ID	0.221 0.462	0.471 0.680	1.5	90	09:30
		Hour	33	0.088	0.148	0.699							
		Hour^2	33	−0.028	0.201	0.653							
	Floral visitors (number)	Log(flowers)	42	0.507	2.619	0.105		OLRE Site ID	0.097 0.991	0.311 0.991	0.15	9	08:09
		Hour		0.040	0.036	0.849							
		Hour^2		−0.131	3.838	0.05	*						
Reward secretion	Floral sugar (µg)	Hour	78	0.1445	0.1019	0.7495		OLRE Plant ID	0 0	0 0	0.39	23	08:23
		Hour^2	78	−0.1818	1.3222	0.2502							
	Extrafloral sugar (µg)	Hour	78	0.4647	1.0070	03156		OLRE Plant ID	0.2425 0.4013	0.4925 0.6335	1.13	68	09:08
		Hour^2	78	−0.2053	1.8949	0.1686							
	Pollen deposition (number of grains)	Hour	44	0.3240	21.38	3.73^{-06}	***	OLRE Site ID	0.1049 0.0957	0.3240 0.3094			

*Estimates at which the maxima for mutualist activities and reward production are reached are shown in the last three columns. Peak hour is the time in hours estimated, mpa shows minutes post-anthesis when the maxima is reached, and time of day indicates an approximation of when that activity is likely to occur, although this varies depending on the season and the time of sunrise. *P < 0.05; ***P < 0.001.*

On average, a flower and its associated leaf secreted a total of 2,815 ± 767 µg of sugar via floral and extrafloral nectar throughout the 4.5 h anthesis period. The total sugar content in FN was 149 ± 19.3 µg of sugar, whilst total extrafloral sugar was 2,665 ± 765 µg. Thus, the relative sugar contributions of floral and extrafloral nectar in a leaf-flower module were 5.3 and 94.7%, respectively.

Floral and extrafloral nectaries of *T. velutina* are both able to quickly replenish nectar after experimental removal by non-destructive sampling of the same flowers over the entire anthesis period (**Figure 2A**). FN and EFN are secreted simultaneously during anthesis and with the highest amount of sugar content secreted during the first 2 h of anthesis (**Figure 2A**), although their secretion peaks predicted from model estimates are slightly mismatched (**Table 1**). EFN secretion peaked 68 min after our first collection, which was taken as soon as flowers were fully open, whilst FN peaked 23 min after the first collection. The timing of peaks in secretion of the two types of

nectar matches that for the mutualists that harvest each resource. Peak pollen deposition occurred at the beginning of anthesis and steadily declined over time (**Figure 2C**). Hence, pollen deposition data were analyzed using a linear model without fitting hour as a squared term and we did not estimate timing of daily maxima using model derivations (**Table 1**).

We recorded 1,535 ant visitors from nine ant species patrolling extrafloral nectaries of *T. velutina* at CICOLMA (**Table 2**). *Dorymyrmex bicolor, Paratrechina longicornis*, and *Brachymyrmex* spp. accounted for 68.5% of the total ants observed, and 77.35% of the patrolling ants, after excluding *Monomorium* spp. that were never observed displaying patrolling behaviors on *T. velutina* and are mostly parasitic consumers of FN and EFN (lestobiotic) (Ettershank, 1966; Bolton, 1987).

Direct Conflict

We recorded 991 floral visitors, of which 907 (91.5%) were by the honeybee *Apis mellifera*. Of the remaining 8.5%, 61 visits were by native bees, 11 by flies (Diptera), 10 visits by Lepidoptera, one visit by a beetle (Coleoptera) and one by a wasp (Hymenoptera) (**Table 2**).

Neither the presence of ants inside flowers nor their identity had any significant effect on the number of honeybees visiting the flowers (**Figure 3A**, **Table 3**). The presence of the most aggressive ant species, *Dorymyrmex bicolor*, increased the likelihood of a pollinator displaying inspection behaviors by 20% (**Figure 3B**), and increased by 12% the proportion of time per visit spent displaying inspection behaviors rather than foraging or pollinating the flower (**Figure 3C**). Finally, the presence of *Dorymyrmex bicolor* and *Paratrechina longicornis* inside the flowers halved the duration of contact visits compared to control flowers without ants, or to flowers with *Brachymyrmex* sp. ants inside.

Indirect Conflict

Clogging extrafloral nectaries on the leaves associated with newly emerged floral buds had no effect on their FN volume [$LRT_{(1,49)} = 0.21$; $P = 0.64$, **Table 3**, **Figure 4B**] or sugar content [$LRT_{(1,49)} = 0.087$; $P = 0.77$, **Table 3**, **Figure 4A**]. Differences in FN volume and sugar content ($FN_{post-treatment} - FN_{pre-treatment}$) were positive in plants under both control and clogged treatments (**Figure 4**).

DISCUSSION

Our findings show that both ants and pollinators are active while flowers are open (**Figure 2B**), that FN and EFN are simultaneously secreted (**Figure 2A**), and that pollen deposition occurs when ants are actively patrolling (**Figure 2C**). Consequently, in *T. velutina* guarding ants and pollinators operate in close spatial and temporal proximity, implying that direct and indirect conflict could occur in this system. We found, however, no evidence for indirect, nectar-mediated, conflict between ants and pollinators, since plants did not reallocate resources toward floral nectar, even when 95% of the sugar investment during anthesis, which is in EFN, is prevented (**Figure 2**). We found evidence for direct conflict, as the presence of dead individuals of patrolling ant species inside flowers was associated with both higher frequency of inspection behaviors in potential pollinators, and reduced visit duration (time spent inside flowers) (**Figure 3**). Taken together, these effects increased handling time per flower and reduced pollinator foraging efficiency. Nonetheless, this result was obtained under an experimental setting using dead ants placed inside flowers. Further studies are required to test (i) the effect of living patrolling ants on pollinator visitation, and (ii) the impact of any such effects on plant fitness. The latter are crucial to understanding whether there is ongoing

TABLE 2 | Abundance and identity of the ants recorded patrolling extrafloral nectaries during the 2014 census and the floral visitors recorded during the direct conflict experiment in 2016 on Turnera velutina plants.

	Taxon	Visitors	Percentage	Subfamily	Patrolling
Ants at extrafloral nectaries	*Paratrechina longicornis*	487	31.72	Formicinae	Gregarious
	Dorymyrmex bicolor	421	27.42	Dolichoderinae	Gregarious
	Monomorium ebenium	270	17.58	Myrmicinae	Gregarious
	Camponotus planatus	184	11.98	Formicinae	Loner
	Brachymyrmex sp.	73	4.75	Formicinae	Gregarious
	Camponotus mucronatus	60	3.90	Formicinae	Loner
	Crematogaster sp.	23	1.49	Myrmicinae	Gregarious
	Camponotus novogranadensis	15	0.97	Formicinae	Loner
	Pseudomyrmex gracilis	2	0.13	Pseudomyrmicinae	Loner and very rare
Floral visitors	*Apis mellifera*	907	91.5		
	Native bees	61	6.05		
	Diptera	11	1.10		
	Lepidoptera	10	1		
	Coleoptera	1	0.1		
	Wasps	1	0.1		

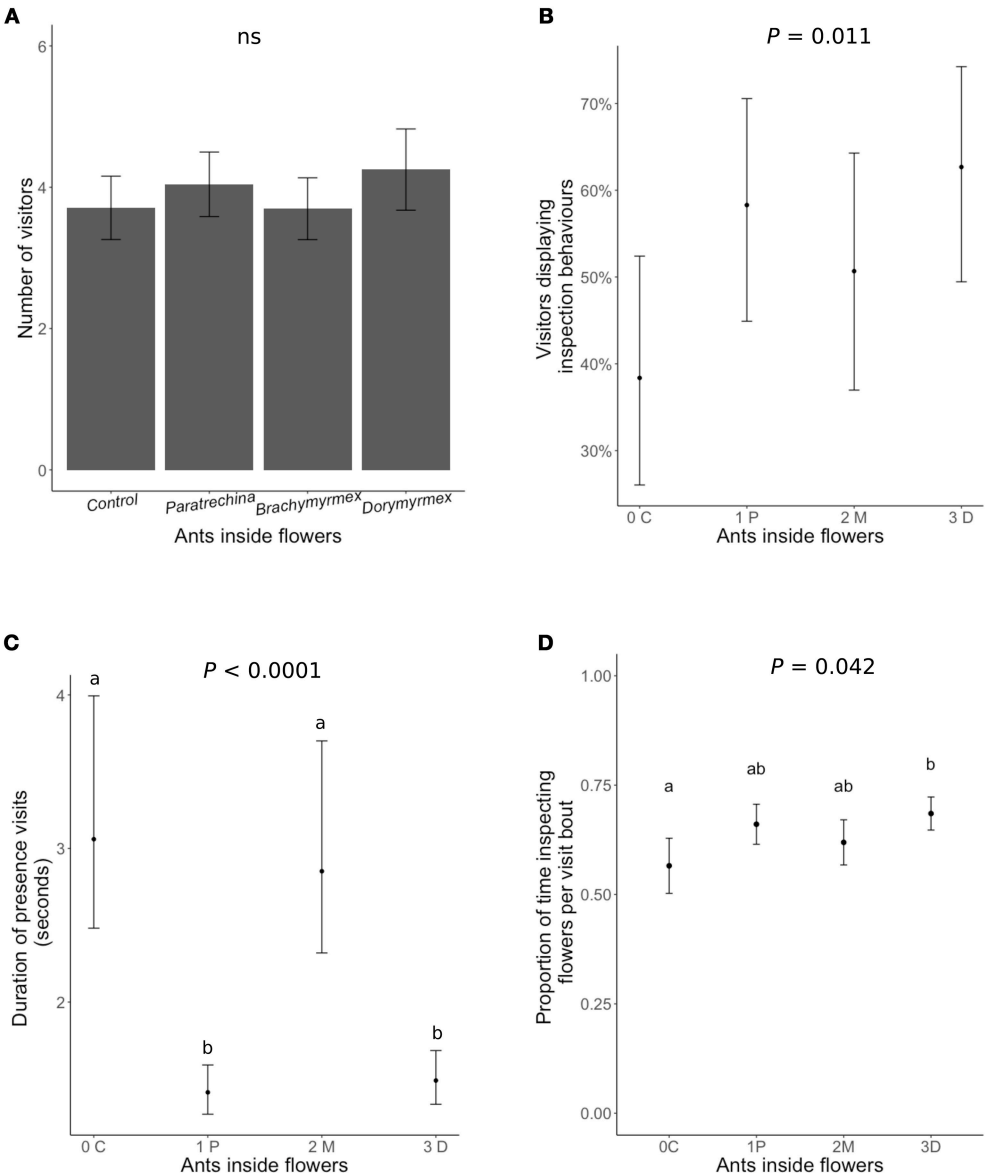

FIGURE 3 | The effect of dead ants inside the flowers of *Turnera velutina* on different aspects of the behavior of visiting honeybees: **(A)** visitation frequency, **(B)** the percentage of visitors of displaying inspection behaviors, **(C)** the duration of contact visits (time spent inside the flower), and **(D)** proportion of time spent inspecting the flowers per visit bout (hovering over the floral head space). Ant species are arranged in order of increasing aggressivity and names are abbreviated: C, control with no ants; 1P, *Paratrechina longicornis*; 2M, *Brachymyrmex* sp.; 3D, *Dorymyrmex bicolor* ants. ($n = 40$ plants; $n = 160$ flowers).

selection on *Turnera velutina* to manage direct ant-pollinator conflict.

Mutualist Activity, Reward Secretion and Pollen Deposition

Floral and extrafloral nectar were secreted simultaneously and rapidly replenished in *T. velutina*, especially during the first 2 h post anthesis (**Figure 2**). Replenishment is a general feature of EFN secretion (Pacini et al., 2003; Pacini and Nepi, 2007; Pacini and Nicolson, 2007). In fact, we are unaware of any report documenting extrafloral nectaries incapable of replenishing secretion after consumption (Pacini et al., 2003; Pacini and Nepi, 2007; Pacini and Nicolson, 2007; Escalante-Pérez and Heil, 2012; Orona-Tamayo et al., 2013; Heil, 2015). In contrast, species vary in whether FN is replenished or not (for details on floral nectar dynamics see: Pacini et al., 2003; Nicolson et al., 2007; Willmer, 2011). We suggest that rapid resupply is crucial in short-lived flowers, such as the one-day flowers of *Turnera* species, because it makes the flower attractive again for another visit, potentially increasing pollen transfer, pollen deposition, and seed set. Interestingly, Dutton et al. (2016) reported no FN resupply in flowers from three congeners (*Turnera ulmifolia,*

TABLE 3 | Model statistics for the experiments testing indirect (nectar-mediated) and direct (pollinator deterrence) ant-pollinator conflict.

	Response	Distribution	Fixed effects	N	LRT	*P*-value		Random effects	Variance	SD
Indirect conflict	Floral nectar (μl)	Gaussian	Clogging treatment	50	0.0813	0.7754		Family	0.6987	0.8359
	Floral nectar (μl)	Gaussian	Clogging treatment	50	0.21006	0.6467		Family	138.5	11.77
Direct conflict	Number of visitors	Poisson	Ants in flowers	95	1.1848	0.7567		OLRE Plant	2.77^{e-08} 0.144	0.0001 0.3803
	Likelihood of being alerted	Binomial	Ants in flowers	373	10.715	0.01337	*	ID visitor Plant	8.279^{e-10} 0.595	2.877^{e-05} 0.7715
	Proportion of time per visit spent displaying inspection behaviors	Gaussian (logit)	Ants in flowers	112	7.5728	0.055	*	ID visitor Plant	0.0009313 0.0201	0.03052 0.14198
	Duration of presence behaviors (sec)	Gamma	Ants in flowers	307	392.37	2.2^{e-16}	***	OLRE ID visitor Plant	0.1202 0.001587 1.928^{e-10}	0.3468 0.03984 0.001389

*$P < 0.05$; ***$P < 0.001$.

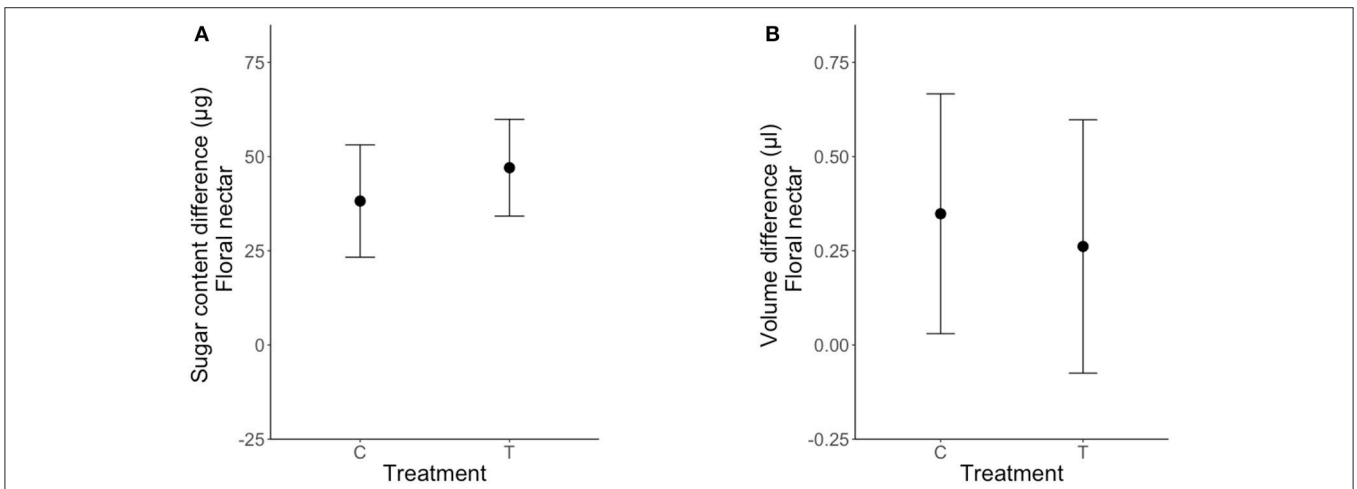

FIGURE 4 | Effect of clogging on nectar secretion and sugar re-allocation in *Turnera velutina* for nectar volume **(A)** and sugar content **(B),** between control (C) and treatment (T) plants (means ± se; $n = 50$ plants). Values shown are a difference in floral nectar, defined as: [FN post-treatment–FN pre-treatment].

Turnera subulata, and *Turnera joelii*), when sampling FN in the morning and afternoon, finding no FN secretion in the afternoon collection. These observations show either variation in nectar resupply within closely related species displaying similar floral biology, or perhaps suggest that shorter term dynamics in the nectar supply of the other three species were not detected by the sampling methods used. The latter highlights the need to test floral resupply within short time scales, because over long sampling intervals, flowers can both secrete and reabsorb nectar (Pacini et al., 2003; Nicolson et al., 2007; Willmer, 2011). Finding no standing crop when a flower is resampled in the afternoon does not rule out replenishment after emptying in the morning, and then reabsorption later in the day (Kearns and Inouye, 1993).

Model-based estimation of the daily timing of the maxima of nectar secretion suggest that floral nectar secretion peaks a few minutes after the corolla is fully open (**Table 3**) and 45 min before peak EFN sugar secretion (**Figure 2A**). This represents a slight mismatch in the timing of rewards for ants and pollinators, which may underlie the 85 min mismatch in estimated peaks of ants and floral visitor activity (**Table 1**). To our knowledge, temporal segregation in the activity of ants and pollinators has been reported only for obligate (myrmecophytic) species patrolled or tended by a single ant species at a time (*Humboltia brunonis* (Fabaceae; Gaume et al., 2005), *Hirtella physophora* (Chrysobalanaceae; Malé et al., 2015), and *Opuntia imbricata* (Cactaceae; 2014)). In some specialized systems, ant and pollinator activity occurs in close proximity and simultaneously, but conflict is prevented by ant-repellent floral volatiles [*Vachellia zanzibarica* (Fabaceae; Willmer and Stone, 1997)] and *Vachellia hindsii* (Fabaceae; Raine et al., 2002). On the other hand, temporal overlap in

ant activity at extrafloral nectaries and pollinator visitation to flowers has been reported for facultative ant-plants associated with many ant species simultaneously [*Vachellia constricta* (Fabaceae; Nicklen and Wagner, 2006), *Acacia myrtifolia*, (*Acacia sensu stricto*, Fabaceae; Martínez-Bauer et al., 2015), *and Heteropterys pteropetala* (Malpighiaceae; Assunção et al., 2014)]. Our results add *Turnera velutina* to the list of facultative myrmecophiles with synchronized ant and pollinator activity (**Figure 2**). This synchronous myrmecophile vs. segregated myrmecophyte pattern is consistent with evidence that ants in obligate mutualisms are more aggressive and better defenders (Chamberlain and Holland, 2009; Rosumek et al., 2009; Trager et al., 2010), but may impose greater ecological costs on host pollination. We suggest that temporal segregation of mutualists and/or ant repellent floral volatiles are alternative strategies that reduce such costs. Further studies on the timing of pollinator, ant visitation and ant aggressivity in a wider range of systems are required to test the temporal component of this hypothesis.

Direct Conflict

We showed that dead ants inside the flowers of *T. velutina* have an impact on honeybee behavior (**Figure 3**). Ant presence was correlated with shorter honeybee flower visits (**Figure 3C**), an increase in the proportion of visitors displaying inspection behaviors, and increased duration of inspection behaviors per visiting bout (**Figure 3**). We interpret longer inspection behavior to indicate increased caution in the bees (as previously assumed by: Altshuler, 1999; Ness, 2006; Junker et al., 2007; Assunção et al., 2014; Cembrowski et al., 2014). Our findings are consistent with work on *Heteropterys pteropetala* in which plastic ants inside flowers negatively affected pollination (Assunção et al., 2014). Results for *H. pteropetala* differ from ours in that the bees pollinating *H. pteropetala* showed significantly reduced visitation rates to flowers containing plastic ants. In contrast, honeybees in *T. velutina* did not visit flowers containing ant corpses less frequently than control flowers (**Figure 3A**). In both systems, ants feeding at extrafloral nectaries did not hinder pollination (Assunção et al., 2014; Villamil et al., unpublished data). This suggests that while pollinators avoid ants in flowers, plants may have evolved other mechanisms to prevent ants from entering flowers, resulting in only rare encounters between ants and pollinators.

Although experiments that place ant cues on flowers can tell us about the response of pollinators to ants, they must be interpreted with caution as an indicator of current ant-pollinator conflict. Firstly, because ants may rarely enter flowers (Villamil et al. submitted). Secondly, by placing such ant cues in flowers we may be violating existing ant-excluding or ant-repelling plant mechanisms (Willmer, 2011). Thirdly, in contrast to such experimental treatments, ants do not naturally remain in the flowers for long periods (Assunção et al., 2014), and only a low proportion of flowers may be occupied at any one time. For instance, in *T. velutina* only 10% of the flowers are occupied by ants (Villamil et al., submitted).

Does Herbivore Deterrence Match Pollinator Deterrence?

Although some studies have documented variation among ant species in aggression toward herbivores (Ness, 2006; Miller, 2007; Ohm and Miller, 2014), little is known about the effect of different patrolling ant species with varying levels of aggressivity on pollinator visitation (Ness, 2006; Miller, 2007; LeVan et al., 2014; Ohm and Miller, 2014). Nonetheless, a positive correlation between the level of defense provided and the level of pollinator deterrence they exert has often been assumed since ant traits involved in defense (patrolling activity and aggressivity) are likely to be the same as those involved in pollinator deterrence (Ohm and Miller, 2014). Bees tend to forage in a way that maximizes the net benefit of each foraging trip (Stephens and Krebs, 1986; Jones, 2010; Cembrowski et al., 2014). When foraging in ant-plants, this benefit might be maximized if foragers avoid flowers or patches where predation risk is high (Dukas, 2001; Dukas and Morse, 2003; Ness, 2006; Jones and Dornhaus, 2011; Assunção et al., 2014), as could be the case when encountering ant species that attack pollinators. Some ants also consume FN and pollen, and such plants may represent high risk foraging environments with low net rewards for pollinators (Ness, 2006). Shorter or fewer visits to such flowers may be a pollinator strategy to maximize foraging efficiency by avoiding flowers, plants, or patches with high predation risk (Jones and Dornhaus, 2011).

In *T. velutina*, the most aggressive ant guard, *Dorymyrmex bicolor*, had the strongest effect on pollinator behavior (**Figure 3**), while *Brachymyrmex* sp. ants inside the flowers did not reduce the duration of pollinator visits. The least effective anti-herbivore ant species, *Paratrechina longicornis* (Villamil unpublished data), halved the duration of pollinator visits (**Figure 3**). In *Ferocactus wislizeni*, plants tended by *Solenopsis xyloni*, the most aggressive ant species, had fewer and shorter pollinator visits (Ness, 2006). Such differences are consistent with pollinator sensitivity to ant aggressiveness. In contrast, although ant exclusion in *Opuntia imbricata* significantly increased pollinator visitation, there were no differences in impacts associated with different ant species (Ohm and Miller, 2014), and no evidence that the more aggressive guard (*Liometopum apiculatum*) had a stronger deterring effect on pollinators (Ohm and Miller, 2014). Whether the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence remains unknown (but see: Ness, 2006; Miller, 2007; LeVan et al., 2014; Ohm and Miller, 2014), and should be tested, not assumed.

Indirect Conflict

Our experimental approach found no evidence for a trade-off in sugar investment in extrafloral and floral nectar in *T. velutina*. We conclude that there is no indirect nectar-mediated conflict between guarding ants and pollinators in *Turnera velutina*, and that pollinators do not obtain greater rewards when rewards for patrolling ants are eliminated.

We found only two previous studies testing indirect, nectar-mediated ant-pollinator conflict by quantifying sugary rewards

(FN and EFN) to both mutualists (Chamberlain and Rudgers, 2012; Dutton et al., 2016). Previous work on other *Turnera* species by Dutton et al. (2016) found evidence of a trade-off in two of the three *Turnera* species tested; removing EFN decreased FN and *vice versa* in *T. ulmifolia* and *T. subulata*, but not in *T. joelii*. Interestingly, both *Turnera* species in which trade-offs were detected by Dutton et al. (2016) invested equally in FN and EFN, whilst *T. joelli* (which showed no trade-off) invested more in EFN (Dutton et al., 2016). The same pattern holds for *T. velutina*, a species with an asymmetric investment toward EFN, which accounts for 95% of the sugar allocation per leaf-flower module, and where we found no trade-off or resource reallocation from EFN to FN (**Figure 4**). Unfortunately data on FN and EFN volume and sugar content were not reported for the cotton species (Chamberlain and Rudgers, 2012).

One possible reason for lack of a trade-off is that sugar is not a limiting resource for the plant. If so, there would be no reason to expect dynamic reallocation. Estimates of the metabolic costs of nectar secretion vary, and while some studies suggest low metabolic costs (O'Dowd, 1979: EFN accounts for 1% of the total energy invested per leaf), others indicate investment of up to 37% of daily photosynthesis in floral nectar (Southwick, 1984; Pyke, 1991). A second reason, which applies in particular to comparative cross-species analyses rather than experimental manipulations, is that investment in both forms of nectar may be influenced by other aspects of life history strategy. Chamberlain and Rudgers (2012) found no significant negative correlations between extrafloral nectary and floral traits in a comparative analysis across cotton (*Gossypium*) species, and correlations were significantly positive in 11 of 37 cotton species. Foliar extrafloral nectary volume was positively associated with plant investment in floral nectar, rejecting the hypothesis of trade-offs among investments in pollinators versus bodyguards in *Gossypium*. Several potential mechanisms underlying the positive correlations between FN and EFN have been proposed, including pleiotropy, and genetic, physiological or ecological linkage (Chamberlain and Rudgers, 2012). The pleiotropy or genetic linkage hypothesis could be tested using genome sequencing (Chamberlain and Rudgers, 2012). Positive correlations could also arise from physiological or ecological linkage. Traits such as FN and EFN may be physiologically linked. However, the fact that in *Gossypium* FN volume was most strongly correlated with foliar EFN volume, but FN was weakly correlated with bracteal EFN volume (Chamberlain and Rudgers, 2012) questions the physiological linkage hypothesis since bracteal and floral nectaries are spatially closer than floral and extrafloral nectaries, but they are not strongly correlated. We suggest that lack of any trade-off could also indicate that FN and EFN may be phenotypically integrated as a functional module for mutualist attraction. Although formal analyses are required to test this hypothesis, we think it is a strong possibility since *T. velutina* leaves are phenotypically integrated modules in which leaf economics, defensive and morphological traits covary and are ecologically linked (Damián et al., 2018). Whatever the drivers of these positive correlations may be, available evidence suggests that plants may experience fewer investment trade-offs among different functional traits than previously assumed.

CONCLUSIONS

To our knowledge, trade-offs between extrafloral and floral nectar traits have been studied in 41 species from two genera: 37 *Gossypium* species (Chamberlain and Rudgers, 2012), and four *Turnera* species, including this study (Dutton et al., 2016; Villamil, 2017, **Figure 2**). Negative correlations or evidence for trade-offs have been found in only two of these species: *T. ulmifolia* and *T. subulata* (Dutton et al., 2016), representing less than 5% of the species studied. Although many more studies are required to shed light on quantitative trends of floral and extrafloral investment in plants, trade-offs between floral and extrafloral seem infrequent. On the other hand, evidence of direct conflict with patrolling ants reducing pollinator visitation frequency and duration, inducing inspection behaviors and increasing foraging time has been widely reported (Rudgers and Gardener, 2004; Ness, 2006; Chamberlain and Rudgers, 2012; Malé et al., 2012, 2015; Assunção et al., 2014; Koptur et al., 2015; Martínez-Bauer et al., 2015). We suggest that these two issues are not isolated, and hypothesize that positive correlations between FN and EFN investment in ant-plants may be a plant strategy to compensate or lure pollinators to apparently risky flowers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors upon request.

AUTHOR CONTRIBUTIONS

NV conceived the ideas, all authors designed the experiments. NV conducted fieldwork, processed, and analyzed the data. NV led the writing of this paper, with critical inputs from KB and GS.

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Crop Domestication Alters Floral Reward Chemistry With Potential Consequences for Pollinator Health

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Crop domestication can lead to weakened expression of plant defences, with repercussions for herbivore and pathogen susceptibility. However, little is known about how domestication alters traits that mediate other important ecological interactions in crops, such as pollination. Secondary metabolites, which underpin many defence responses in plants, also occur widely in nectar and pollen and influence plant-pollinator interactions. Thus, domestication may also affect secondary compounds in floral rewards, with potential consequences for pollinators. To test this hypothesis, we chemically analysed nectar and pollen from wild and cultivated plants of highbush blueberry (*Vaccinium corymbosum* L.), before conducting an artificial diet bioassay to examine pollinator-pathogen interactions. Our results indicated that domestication has significantly altered the chemical composition of *V. corymbosum* nectar and pollen, and reduced pollen chemical diversity in cultivated plants. Of 20 plant metabolites identified in floral rewards, 13 differed significantly between wild and cultivated plants, with a majority showing positive associations with wild compared to cultivated plants. These included the amino acid phenylalanine (4.5 times higher in wild nectar, 11 times higher in wild pollen), a known bee phagostimulant and essential nutrient; and the antimicrobial caffeic acid ester 4-*O*-caffeoylshikimic acid (two times higher in wild nectar). We assessed the possible biological relevance of variation in caffeic acid esters in bioassays, using the commercially available 3-*O*-caffeoylquinic acid. This compound reduced *Bombus impatiens* infection by a prominent gut pathogen (*Crithidia*) at concentrations that occurred in wild but not cultivated plants, suggesting that domestication may influence floral traits with consequences for bee health. Appreciable levels of genetic variation and heritability were found for most floral reward chemical traits, indicating good potential for selective breeding. Our study provides the first assessment of plant domestication effects on floral reward chemistry and its potential repercussions for pollinator health. Given the central importance of pollinators for agriculture, we discuss the need to extend such investigations to pollinator-dependent crops more generally and elaborate on future research directions to ascertain wider trends, consequences for pollinators, mechanisms, and breeding solutions.

Keywords: domestication, floral rewards, *Vaccinium*, crop evolution, pollinator-pathogen interactions, *Bombus impatiens*, pollinator health, phytochemicals

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INTRODUCTION

Humans have domesticated and selectively bred crops for millennia (Smith, 2001). While domestication has delivered many agronomic benefits, including enhanced yield and other desirable traits (Evans, 1996; Bai and Lindhout, 2007; Dempewolf et al., 2017), negative or unintended consequences of this process have included reduced genetic diversity (Van de Wouw et al., 2010; Meyer et al., 2012), and the impaired function or disappearance of potentially useful traits (Rosenthal and Dirzo, 1997; Li et al., 2017) such as physical or chemical defences against herbivory and disease (Wink, 1988; Jones, 1998; Gols et al., 2008; Sujana et al., 2012). Plant defence traits can impose considerable resource allocation costs, and can thereby trade-off with fitness or yield (Kempel et al., 2011). Hence, where selection is consistently imposed over many generations to maximise crop yield, agronomic gains may accrue at the risk of compromising other traits from which resources are diverted (Rosenthal and Dirzo, 1997; Brown and Rant, 2013). It is only relatively recently, however, that the extent of domestication impacts on crop ecological function has come to be fully appreciated (Meyer et al., 2012; Chen et al., 2015; Whitehead et al., 2017). This topic has garnered increased attention due to growing interest in the agronomic potential of traits traditionally neglected in crops, such as herbivore-induced plant volatiles (HIPVs) and extrafloral nectaries involved in the attraction of pest natural enemies (Rasmann et al., 2005; Rodriguez-Saona et al., 2009; Chen et al., 2015; Heil, 2015; Li et al., 2017).

In contrast to impacts on crop interactions with herbivores and pathogens, very little is known about the effect of domestication on interactions with pollinators and the traits which mediate these interactions. What little is known on this topic to date has mostly come from the study of ornamental plants, where widespread loss of floral scent is a reported consequence of breeding (Vainstein et al., 2001; Pichersky and Dudareva, 2007). This lack of information on domestication effects on pollinator-relevant traits, including floral reward chemistry, is surprising, given the importance of insect pollination for agriculture. About 75% of the world's staple food crops rely to some extent on the ecosystem service of pollination (Klein et al., 2007), which contributes an estimated $351 billion (USD) annually to global food production (Lautenbach et al., 2012). Pollinators also have the potential to contribute to human nutritional health and need for micronutrients (Chaplin-Kramer et al., 2014; Ellis et al., 2015), and improve global food security (Bailes et al., 2015).

Although historically studied separately, plant-herbivore and plant-pollinator interactions are interconnected (Strauss and Armbruster, 1997; Adler et al., 2001; Irwin et al., 2003; Strauss and Irwin, 2004; Kessler and Halitschke, 2009; Lucas-Barbosa, 2016; Muola et al., 2017; Nepi et al., 2018). For example, a broad range of defence-related secondary metabolites (including alkaloids, phenolics, and cyanogenic glycosides) are expressed in floral rewards, including nectar and pollen (Baker, 1977; Rhoades and Bergdahl, 1981; Adler, 2000; Adler and Irwin, 2005; Heil, 2011; Arnold et al., 2014; Irwin et al., 2014; Stevenson et al., 2017). These compounds range widely in concentration – from trace amounts, to concentrations similar to other plant tissues (Stevenson et al., 2017, and references therein) – and in ecological function. The ecological effects of secondary compounds in floral rewards include warding off nectar robbers and microbial growth (Irwin et al., 2004; González-Teuber and Heil, 2009; Barlow et al., 2017), enhancing pollinator memory (Wright et al., 2013), and reducing pollinator infection by pathogens and parasites (Baracchi et al., 2015; Richardson et al., 2015; Erler and Moritz, 2016; Palmer-Young et al., 2016). Evidence now suggests that secondary metabolites occur ubiquitously in floral rewards, and commonly show phenotypic integration across herbivore- and pollinator-consumed materials in wild and cultivated plants (Adler et al., 2006, 2012; Kessler and Halitschke, 2009; Manson et al., 2012; Cook et al., 2013; Richardson et al., 2016; Palmer-Young et al., in press). It is therefore of great interest to assess whether domestication effects on plant defence are also mirrored for floral reward chemistry, with potential ramifications for ecological function.

We used blueberry as a crop system to examine the effect of domestication on floral reward chemistry and its potential consequence for pollinators. We included in this study wild and cultivated plants of *Vaccinium corymbosum* L. (highbush blueberry; Ericaceae), including cultivated hybrid and introgressed crosses with *V. angustifolium* Ait. (lowbush blueberry). The relatively recent and well-documented history of domestication of *Vaccinium* species (including cranberry, lingonberry, and bilberry) has rendered the genus a useful model for the study of domestication effects (Rodriguez-Saona et al., 2011; Song and Hancock, 2011; Rivera et al., 2015, 2016; Hernandez-Cumplido et al., 2018). The history of domestication of highbush and lowbush blueberry dates back to 1908 and 1909, respectively, when the first wild plants were collected from Greenfield, New Hampshire, United States, for cultivation and selective breeding (Gough, 1993). This initial highbush collection, together with two additional wild selections from New Jersey (Ehlenfeldt, 2009), formed some of the first early cultivars of *V. corymbosum* ('Brooks,' 'Sooy,' 'Rubel'). Together with their crosses ('Katherine,' 'Pioneer'), these first selections continue to make the largest genetic contributions to modern highbush cultivars (Ehlenfeldt, 1994; Lobos and Hancock, 2015), which form the basis of the global blueberry industry.

We examined nectar and pollen chemistry in eight cultivars or interspecific crosses of *V. corymbosum*, and as a basis for comparison, sampled from three wild populations within ca. 40 km of where the original founding cultivar 'Brooks' was collected. To examine potential domestication effects on pollinator health via changes in the pathogens of pollinators, we conducted an artificial diet bioassay with the common eastern bumble bee, *Bombus impatiens* Cresson. Bees were infected with the pathogen *Crithidia*, before manipulating dietary levels of a caffeic acid ester at concentrations representative of wild and cultivated plant nectar to compare their effect on infection. *Crithidia* species are Trypanosomatid gut pathogens that infect *Bombus* species via faecal-oral contamination (Durrer and Schmid-Hempel, 1994). Infection by *Crithidia* can reduce learning (Gegear et al., 2005, 2006), colony growth rates (Shykoff and Schmid-Hempel, 1991) and queen founding

success (Brown et al., 2003), and is associated with reduced reproduction in wild bumble bee colonies (Goulson et al., 2018). In Massachusetts, United States *Crithidia* can infect up to 80% of *Bombus* (Gillespie, 2010).

Specifically, we tested the following hypotheses: (1) Domestication has altered chemical composition and reduced chemical diversity of nectar and pollen; (2) Nectar and pollen chemical traits show genetic variation and heritability, but cultivars are phenotypically less variable than wild populations, consistent with their clonal propagation; (3) Nectar and pollen chemical concentration and composition are genetically correlated, which could constrain targeted breeding for floral reward chemistry; and (4) Altered expression of nectar antimicrobial compounds, such as caffeic acid esters, can influence pollinator-pathogen interactions.

MATERIALS AND METHODS

Plant Material and Field Sampling

Eight cultivars were selected for inclusion in this study, based on suitable replication across sampled farms: 'Patriot' (P), 'Reka' (R), 'Liberty' (L), 'Bonus' (B), 'Friendship' (F), 'Northland' (N), 'BlueCrop' (BC), and 'Spartan' (S). The genetic origin of the majority of these cultivars is pure *V. corymbosum* (cultivars B, L, N, R, and S), whereas cultivars BC, F, P are interspecific hybrids with minor parentage from *V. angustifolium* (**Supplementary Table 1**). A range of ploidy levels are known to exist within the genus *Vaccinium*. However, commercially grown highbush cultivars, including those with minor percentages of *V. angustifolium*, are reported to be tetraploids (Rowland and Hammerschlag, 2005). The cultivars included in this study, and domesticated highbush plants generally, are typically propagated by vegetative means (softwood or hardwood cuttings) (Gough, 1993), meaning that all sampled plant individuals of the same cultivar were genetic clones.

Nectar and pollen from wild and cultivated highbush blueberry were sampled in western Massachusetts, United States, in May 2014. Wild samples were collected from three sites at least 5 km apart between May 15 to 26 [Harvard Forest (HF): 42°32′6″N, 72°11′19″W, Harvard Pond (HP): 42°29′54″N, 72°12′47″W, and Quabbin Reservoir (Q): 42°23′32″N, 72°24′11″W]. We sampled cultivars at several farms between May 19 and 28 from Cold Spring Orchard (42°15′6″N, 72°21′38″W; cultivars P, R, L, B, F, and N), Kenburn Orchard (42°36′43″N, 72°39′18″W; cultivar P), Nourse Farms (42°25′49″N, 72°35′18″W; cultivars BC and S) and Sobieski's River Valley Farm (42°27′12″N, 72°35′43″W; cultivars BC and S). To ensure no large confounding effect of sampling across multiple farms, cultivar-by-farm interactions were tested but were not significant (data not shown) for nectar and pollen chemical composition and diversity, as quantified below. For analyses other than nectar and pollen chemical composition and diversity, we included only cultivars which had been sampled from a single farm (Cold Spring Orchard).

Ten wild plants were sampled per site, and 5 cultivated plants per cultivar per farm. To prevent pollinator access to nectar, flowers were bagged with mesh usually one day before collection for all plants except at Sobieski's farm, where we obtained sufficient nectar without bagging; sometimes bags were left on plants for several days if weather was not conducive to collection (e.g., rain). Where a mixture of bagged and unbagged samples were collected for a cultivar (i.e., BC and S), we tested to affirm that bagging did not hold a significant effect on compound concentrations (data not shown). Nectar was typically collected from flowers using 2 μl glass microcapillary tubes inserted into the flower. Nectar was pooled across flowers within plants with a target of 20 μl per plant (range: 6.76–32.16 μl), and added to 80 μl ethanol in a 1.5 ml microcentrifuge tube. Anthers were collected using forceps, and thus our samples included the pollen sac and a small amount of filament in addition to granular pollen. We collected at least 5 mg per plant by pooling across flowers within plants. Nectar and pollen samples were placed on ice in a cooler in the field, frozen at −20°C, and then freeze-dried prior to analysis.

Chemical Analysis

Freeze-dried nectar was redissolved in 50 μL MeOH. For extraction of pollen, dried samples were sonicated for 10 min in 1 ml MeOH, and incubated at room temperature for 24 h. Samples were then centrifuged at 13,000 rpm for 5 min, and supernatant transferred to glass vials for analysis. Sample analyses were performed by liquid chromatography electrospray ionisation mass spectroscopy (LC-ESIMS) and UV spectroscopy using a Micromass ZQ LC-MS (Waters, Elstree, Herts, United Kingdom). Aliquots of nectar were injected directly onto a Phenomenex (Macclesfield, Cheshire, United Kingdom) Luna C18(2) columns (150 × 3.0 mm i.d., 5 um particle size) and compounds eluted using MeOH (A), H_2O (B) and formic Acid (C) with A = 0%, B = 90% at T = 0 min; A = 90%, B = 0% at T = 20 min and held for 10 min with C at 10% throughout the analyses. Column temperature was 30°C with flow rate = 0.5 ml min^{-1}. High resolution MS spectra were used to provide additional data for compound identification and were recorded for a subset of samples using a Thermo LTQ-Orbitrap XL mass spectrometer (Waltham, MA, United States) with compound separation on an Accela LC system using similar elution parameters as described above. Except for identification of caffeoylquininc and caffeoylshikimic acids (see below), compounds were identified by comparison with mass spectra available via the NIST spectral database version 2.0 and with authentic standards available at Royal Botanic Gardens, Kew. Compounds were quantified using calibration curves with authentic standards based on UV absorbance or compound ionisation in the mass spectrometer. Where these were not available, compound quantities were determined using standard curves calculated from the UV absorbance of compounds with the same chromphore.

These methods also permitted the detection of two amino acids, phenylalanine and tryptophan. For convenience, we used the term 'total amino acids' to refer to the two amino acids only detected and quantified. Compound concentrations are for nectar expressed in μmol L^{-1} original volume, and for pollen in

μmol kg^{-1} dry mass. We refer to 3-, 4-, and 5-*O*-caffeoylquinic acids (CQAs) collectively as chlorogenic acids, and specifically to 3-CQA as chlorogenic acid.

Our LC-MS analysis identified CQAs as principal secondary metabolites in nectar based on their mass spectral properties, which were similar to those reported previously for these compounds (Schütz et al., 2004). This identification was supported by comparison with authentic standards from our own in-house (Jodrell Laboratory) collection, and their accurate mass pseudomolecular ion of *m/z* 355.1038 which gave a protonated molecular formula $[M+H]^+$ of $C_{16}H_{19}O_9$. CQAs are esters of caffeic acid with a hydroxylated cyclohexanoic acid, quinic acid (Schütz et al., 2004). Our analysis also identified an additional caffeic acid ester in nectar of *V. corymbosum* which had a similar UV spectrum to the CQAs (indicative of a caffeoyl moiety). However, this ester recorded a pseudomolecular ion of *m/z* 337.0926 (calcd. 337.0918), corresponding to a protonated molecular formula $[M+H]^+$ of $C_{16}H_{17}O_8$, suggesting a dehydro derivative of CQA. Further comparison of the (-) MS2 data showed major fragment ions at *m/z* 291 and 179, indicative of caffeoylshikimic acids as reported in *Phoenix dactylifera* (Habib et al., 2014). Subsequent co-elution with an authentic laboratory standard and (-) MS2 showing similar fragments at 179, 135, 161, and 291 allowed assignment of the compound to 4-*O*-caffeoylshikimic acid (4-CSA).

Bumblebee Infection Assay

As per our chemical analysis, 4-CSA largely differentiated wild and cultivated plants of *V. corymbosum* (see section "Results"), and thus warranted further biological evaluation. We tested chlorogenic acid (3-CQA) as a proxy for this compound, since no commercial source of 4-CSA was available, and different caffeic acid esters typically show equivalent biological activities (Guzman, 2014), likely owing to their common *ortho*-dihydroxy substitution (Stevenson et al., 1993). For example, in bioassays against various species of bacteria and yeast, 3-CQA, 5-CQA, and other caffeic acid esters frequently possessed identical minimum inhibitory concentration (MIC) values (Zhu et al., 2004; Xia et al., 2011; Guzman, 2014).

Bioassays were conducted with workers of *Bombus impatiens* parasitized with *Crithidia*. To test how 3-CQA affected *Crithidia* infection, we experimentally infected *Bombus impatiens* workers before feeding them 30% sucrose solution without or with 3-CQA ($C_{16}H_{18}O_9$; molecular weight 354.31 g/mol; CAS number 327-97-9; Sigma-Aldrich, St. Louis, MO, United States) at two concentrations, low (2.8 μM [1 ppm] 3-CQA dissolved in 30% sucrose solution), and high (56.5 μM [20 ppm] dissolved in 30% sucrose solution). These represented low and high concentrations of 4-CSA found naturally in cultivated (4.44 μM ± 7.52 SD) and wild (22.03 μM ± 27.46 SD) plants, respectively (see section "Results").

Thirty worker bees were tested per treatment (n = 90 bees total), evenly spread across three source colonies (Biobest Canada, Leamington, Canada; 10 bees per source colony per treatment). Bees were removed from their source colonies, randomly assigned to treatments, starved for 4–6 h, and then experimentally infected with *Crithidia* on the same day. *Crithidia* were isolated from wild *B. impatiens* collected in Massachusetts, United States (GPS coordinates: 42°24′25″N, 72°31′46″W), and maintained in *B. impatiens* colonies in the laboratory. *Crithidia* inoculum was prepared according to a standard protocol (Richardson et al., 2015). In brief, on the day of experimental inoculation, 10 workers were sacrificed from the infected colonies and their intestinal tracts ground individually in 300 μl of dH_20. After vortexing the samples, we allowed them to settle at room temperature for 4 h. We then placed 10 μl of the clear solution on a haemocytometer and examined it for *Crithidia*. For samples that contained *Crithidia*, we transferred 50–150 μl of solution to a clean container and diluted to make an approx. 30% sucrose solution with 6000 *Crithidia* cells × 10 μl^{-1}. Each bee was provisioned with 10 μl of inoculum containing 6000 *Crithidia* cells, which is within the range of concentrations of the pathogen that bees encounter (e.g., Schmid-Hempel and Schmid-Hempel, 1993; Otterstatter and Thomson, 2006). Immediately following inoculation, bees were housed individually in plastic containers. They were given one lump of honey bee collected pollen (Koppert Biological Systems, Howell, MI, United States) mixed with sugar water, and new sugar solution *ad libitum* of the appropriate treatment. Pollen and nectar were replaced daily for 7 days.

After 7 days, we sacrificed individuals and prepared intestinal tracts as when making *Crithidia* inoculum. We placed 10 μl of intestinal solution on a haemocytometer and counted the number of *Crithidia* cells in five subfields of the grid at 10x magnification, totalling 0.02 μl, with a dissecting microscope. We summed these counts and calculated the number of *Crithidia* cells per mL for each bee. We also removed the right forewing of each bee, mounted it on a microscope slide, and measured the radial cell length as an estimate of bee size (Harder, 1982).

Statistical Analyses

Chemical Composition

Comparisons of chemical composition between wild and cultivated plant nectar and pollen were made on both an absolute and proportional basis, which describe two different aspects of composition. Hence, the quantitative concentration of compounds was used directly in the absolute composition analysis, whereas the relative concentration of compounds (their percent contribution on a molar basis relative to other compounds in the sample) was used in proportional composition analysis. For this, a proportional dataset was generated in which compound concentrations within a sample row were standardised by dividing each by the row total. Absolute composition was examined by plotting principle components analysis (PCA) ordinations using the 'rda' function in the R package 'vegan' (Oksanen et al., 2017). PCA provided good representation of the multivariate composition of nectar and pollen, in which the cumulative proportion of variance explained by the first two components was 0.84 and 0.65, respectively. For proportional analyses, unconstrained NMDS (Non-metric multidimensional scaling) ordinations were fitted and plotted based on a Bray-Curtis distance metric in 'vegan.'

As a complement to ordinations, the hypothesis that wild and cultivated plants differed in nectar and pollen chemical composition was explicitly tested using permutational multivariate ANOVAs (function 'adonis' in vegan). For this, distance matrices were first generated for absolute composition based on Euclidean distance (to better preserve quantitative relationships), and for proportional composition using Bray–Curtis distance. Similar to univariate ANOVA, this technique relies on the assumption of multivariate homogeneity between groups (i.e., between wild and cultivated plants). According to homogeneity of multivariate dispersions tests (vegan function 'betadisper'), this assumption was met for all variables except nectar proportional composition. As an alternative to permutational multivariate ANOVA, we therefore opted to compare nectar proportional composition based on axis scores extracted from NMDS ordination. NMDS axis 1 and 2 scores provided a reduced dimensionality representation of proportional composition, and were each fitted as a univariate response in generalised linear mixed models (GLMMs) using the R package 'nlme' (Pinheiro et al., 2017). The GLMMs were fitted with an explanatory variable of 'origin' (wild or cultivated), and a nested random effect of group ID (cultivar or population name). A variance structure was added to both models via the 'weights' function of nlme, to control for unequal variances between wild and cultivated plants. Models were otherwise validated by examining standardised residuals for normal distribution.

'Indicator compound analysis' (or multilevel pattern analysis) was implemented using R package 'indicspecies' (Cáceres and Legendre, 2009) to further probe any potential differences in chemical composition between wild and cultivated plants. This analysis used a permutational approach to test for compound association (or differences in abundance) between different groups (De Cáceres, 2013), in our case wild and cultivated plants. Compound association values (i.e., point-biserial correlation coefficients) and their corresponding *p*-values (testing the null hypothesis that the correlation is zero) were generated using the 'multipatt' function, based on 999 permutations, and use of a corrected correlation coefficient (specified as 'r.g' in multipatt). This correlation coefficient is similar to Pearson correlation, and is likewise bounded between zero and one.

Chemical Diversity

Chemical diversity was assessed both in terms of compound richness (the total number of compounds per sample), and through calculation of a chemical diversity index (H'_C) per sample, following previous authors (Epps et al., 2007; Meier and Bowman, 2008). Calculation of this index used the same equation as for Shannon–Wiener diversity (computed using the vegan 'diversity' function). Hence, H'_C took into account information on compound concentration as well as compound richness. We used linear mixed models (LMMs) to examine potential differences in H'_C between wild and cultivated plants for nectar and pollen. LMMs were fitted using the nlme package, specifying H'_C as a response, 'origin' (cultivated or wild) as a fixed effect, and group ID (cultivar or population name) as a nested random effect. We used ANOVAs to test for significant differences in H'_C between cultivars, as well as between wild populations. All models were validated by checking residuals for equal variance and normal distribution.

Genetic Variation, Correlation and Heritability

We examined genetic variation, genetic correlation, and heritability (as described below) for the six cultivars sampled at Cold Spring Orchard for six traits summarising nectar and pollen chemistry: chemical diversity (H'_C), chemical composition (i.e., NMDS axis 1 scores), total amino acids, total phenolic acids, total flavonols, and total flavan-3-ols. The latter were only included for pollen analyses, as these compounds were not identified in nectar. Chemical composition was quantified via NMDS, as described above. Since our estimates of genetic variation (V_G) and covariance (Cov_G) were based on the use of clones, which therefore included both additive and other genetic effects, these are considered total genetic estimates (de Araújo and Coulman, 2004). We hence report estimates of total genetic variation, total genetic correlation, and broad-sense (or clonal) heritability (H^2) – the genetic contribution to cultivar phenotypic variance. High values of H^2 hence indicate that a focal trait is largely under genetic control, whereas low values mean that most phenotypic variance is due to environmental factors.

Genetic variation and heritability were analysed by fitting variance component (or random effects) models using the R packages 'rptR' (Stoffel et al., 2017), 'lme4,' and its extension 'lmerTest' (Kuznetsova et al., 2017). Each trait was fitted as a univariate response, and 'cultivar' as a random effect. We used the 'rand' function in lmerTest to assess the magnitude and significance of genetic variation, based on the chi-squared (χ^2) statistic, and its corresponding *p*-value from a likelihood ratio test. From the same model, the 'rpt' function in rptR was used to provide estimates of H^2 and its standard error. A bootstrapping procedure (n = 1000 iterations) was implemented within the function to test significance for H^2.

Genetic correlations between traits were analysed using Bayesian multivariate mixed models, implemented in the package 'MCMCglmm' (Hadfield, 2010). For within-material correlations (e.g., between total amino acids and total flavonols), two models were fitted; one which included all nectar traits as a multivariate response, and a second including all pollen traits. For between-material correlations (e.g., between total amino acids across nectar and pollen), a combined model was fitted which included all nectar and pollen traits as a multivariate response. Use of multivariate models in this sense allowed genetic variance (VG) and covariance (Cov_G) components to be estimated for and between traits, respectively. Based on these components, genetic correlation (rG) was calculated for a given set of traits, A and B, as: $rG = Cov_G(A,B)/sqrt[V_G(A), V_G(B)]$. Following Houslay and Wilson (2017), we reported the posterior mean of rG estimates, and assessed the statistical support for rG through plotting 95% credible intervals for the posterior distribution. When this interval did not include zero, we considered genetic correlations to be statistically supported. All traits were fitted as a Gaussian response, aside from nectar total amino acids and total phenolic acids, for which an 'exponential' family was used. For

each model, we employed uninformative parameter-expanded priors for the random effects, and allowed for different trait means, unstructured (co)variances, and trait-specific intercepts. The within-material and between-material models were run for 160,000/1,500,000 iterations, respectively, discarding the first 10,000 iterations as burn-in, and sampling every 75 iterations thereafter. Convergence was assessed by means of the Gelman-Rubin criterion, in which four chains were run to check that they converged to the same posterior distribution. As additional diagnostics, we examined trace and autocorrelation plots for consistent variation and non-autocorrelation between iterations, respectively, and checked that posterior estimates were robust to different starting priors.

As an alternative to direct comparisons of genetic variation in traits between wild and cultivated plants (which was not possible since we have no basis to partition genetic variation from phenotypic variation in wild plants), we compared the phenotypic variability of nectar and pollen composition. Phenotypic variability in chemical composition was measured as the Euclidean distance (or dispersion) of plant individuals from their respective cultivar/population multivariate centroid, following the similar use of this approach in community ecology (Anderson et al., 2006). Hence, to examine whether phenotypic variability differed between wild and cultivated plants, we extracted individual plant distances to their respective group centroids for both nectar and pollen (using the 'betadisper' function in vegan) and fitted these distances as a response variable in LMMs. All other model details then followed the same as for 'chemical diversity' above.

Bumblebee Infection

To test how 3-CQA affected *Crithidia* infection, we used a generalised linear mixed model in SAS version 9.4 with *Crithidia* cells per mL^{-1} (log x+1 transformed) as the response, nectar treatment (control, low or high 3-CQA) as a fixed effect, bee size as a covariate, and bee source colony as a random effect. Tukey's HSD tests were used for *post hoc* pairwise comparisons. Only five bees total (out of 90 bees) died during the course of the experiment, with two deaths in the control treatment, one in the low 3-CQA treatment, and one in the high 3-CQA treatment. These bees were excluded from statistical analysis. Given that so few bees died during the experiment, and that deaths were spread across all treatments, no analysis of mortality was conducted.

RESULTS

Chemical Composition

Across nectar and pollen samples from wild and cultivated plants, we identified 20 compounds belonging to five chemical classes (12 flavonols, 3 caffeic acid esters, 2 amino acids, 2 flavan-3-ols, and 1 norisoprenoid; **Supplementary Table 2**). Nectar contained 9 compounds, pollen 18, and 7 were shared across the material types. Chemical composition of wild and cultivated plants of *V. corymbosum* was highly distinct for nectar (permutational MANOVA: $F = 10.5$, $p < 0.001$) and pollen ($F = 15.0$, $p < 0.001$; **Figures 1A,B**). Indicator compound analysis revealed six nectar compounds and nine pollen compounds which significantly differentiated these groups (**Table 1**). Of these compounds, a majority (4 out of 6 in nectar; 5 out of 9 in pollen) were more positively associated with wild plants, including the caffeic acid ester 4-*O*-caffeoylshikimic acid (4-CSA). In terms of proportional chemical composition, nectar of wild and cultivated plants was moderately distinct (LMM – NMDS axis 1: $F = 0.05$, $p = 0.828$; NMDS axis 2: $F = 13.6$, $p = 0.005$; **Figure 1C**), whereas large differences existed between groups for pollen (permutational MANOVA: $F = 30.0$, $p < 0.001$; **Figure 1C**).

Chemical Diversity

Wild plant nectar consistently lacked the flavonoid glycoside quercitrin, which was abundant in all cultivated plants examined (**Supplementary Table 1**). In contrast, the ester 4-CSA occurred in all wild plants sampled ($n = 30$), but in only 84% of cultivated plants ($n = 55$; **Supplementary Table 2**). As a whole, the chemical diversity index of wild ($H'_C = 1.59 \pm 0.21$ SE) and cultivated ($H'_C = 1.46 \pm 0.13$) plants did not differ significantly (LMM: $t = 0.52$, df = 9, $p = 0.618$; **Figure 2A** inset). Significant differences in nectar chemical diversity index were, however, seen between individual cultivars (ANOVA: $F_{7,47} = 11.76$, $p < 0.001$; **Figure 2A**), but not between wild populations ($F_{2,27} = 0.71$, $p = 0.501$; **Figure 2A**).

Pollen samples of several wild plants contained four flavonoid glycosides absent in cultivated plants (including glycosides of kaempferol, quercetin, and isorhamnetin). However, because these compounds occurred only sporadically (present in only 7-23% of cases; **Supplementary Table 2**), we found no significant difference in pollen chemical diversity index between wild ($H'_C = 2.10 \pm 0.17$ SE) and cultivated ($H'_C = 2.19 \pm 0.22$) plants (LMM: $t = 1.32$, df = 9, $p = 0.220$; **Figure 2B** inset). Similar to nectar, pollen chemical diversity index differed significantly across individual cultivars (ANOVA: $F_{7,46} = 7.94$, $p < 0.001$; **Figure 2B**), but not between wild populations ($F_{2,27} = 2.75$, $p = 0.082$).

Genetic Variation, Correlation, and Heritability

For both nectar and pollen, we observed high levels of total genetic variation and broad-sense heritability among cultivars for most chemical traits (**Table 2**). Notable exceptions included total amino acids in pollen, and total phenolic acids and total flavonols in nectar, which exhibited little to no genetic variation. Weak to moderate genetic correlations were observed between nectar traits, in contrast to mostly strong genetic correlation in pollen traits (**Table 2**). Between-material genetic correlations (conducted for the same trait across nectar and pollen) were non-significant for all traits (**Table 2**). While direct comparison of genetic variation between wild and cultivated plants was not possible, differences in the phenotypic variability of chemical composition were significant for both nectar (LMM: $t = 2.54$, $df = 9$, $p = 0.032$; **Figure 3A** inset) and pollen ($t = 2.59$, $df = 9$, $p = 0.029$; **Figure 3B** inset). Cultivated plants exhibited 62.1 and 47.1% less phenotypic

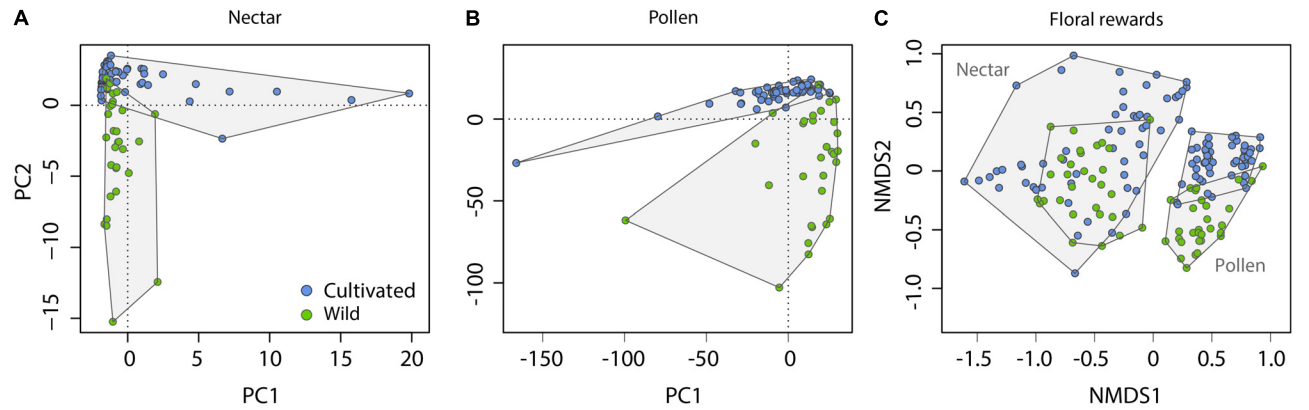

FIGURE 1 | Differentiation of nectar and pollen chemical composition in relation to plant origin (Cultivated vs. Wild). Information on the quantitative concentration of compounds was used to construct PCA ordinations for absolute composition **(A,B)**, whereas relative concentrations of compounds (their percent contribution relative to other compounds in the sample) was used to construct NMDS ordinations for proportional composition **(C)**. Outlying data points in **(A)** are cultivar 'Spartan' (four rightmost points) and 'Northland'; and in **(B)** are cultivar 'Pioneer.'

variation than wild plants for nectar and pollen, respectively. Thus, more variation could typically be found within single wild populations than in the majority of cultivars examined (**Figures 3A,B**).

Bumblebee Infection

Following dietary consumption of 3-CQA by bumble bees parasitised with the gut pathogen *Crithidia*, higher concentrations of 3-CQA significantly reduced infection ($F_{2,79} = 4.15$, $p = 0.020$). Pathogen load was on average reduced by 27% in the high 3-CQA treatment, as compared to a sucrose control or low 3-CQA (**Figure 4**). The covariate of bee size was not related to infection ($F_{1,80} = 0.03$, $p = 0.856$).

DISCUSSION

Our study provides the first evidence that crop domestication can significantly alter floral reward chemistry, and that such

TABLE 1 | 'Indicator compound analysis' of nectar and pollen chemical composition in wild and cultivated plants of *Vaccinium corymbosum*.

	Nectar				Pollen			
	Wild		Cultivated		Wild		Cultivated	
	Value	*p*	Value	*p*	Value	*p*	Value	*p*
Amino acids								
Phenylalanine	0.425	0.009	–	–	0.711	0.018	–	–
Tryptophan	–	–	0.421	0.009	–	–	–	–
Norisoprenoids								
Roseoside	0.609	0.009	–	–	–	–	–	–
Phenolic acids								
4-*O*-caffeoylshikimic acid	0.406	0.009	–	–	–	–	–	–
5-*O*-caffeoylquinic acid	–	–	–	–	–	–	0.378	0.018
Flavonols								
Quercitrin	–	–	0.518	0.009	–	–	–	–
Avicularin	0.438	0.009	–	–	–	–	0.399	0.018
Rutin	–	–	–	–	–	–	0.425	0.018
Quercetin-3-*O*-coumaroylhexoside	–	–	–	–	–	–	0.474	0.018
Quercetin-3-*O*-acetylhexoside	–	–	–	–	0.256	0.022	–	–
Kaempferol-3-*O*-rutinoside	–	–	–	–	0.203	0.050	–	–
Quercetin	–	–	–	–	0.538	0.018	–	–
Isorhamnetin-3-*O*-rhamnosylhexoside	–	–	–	–	0.314	0.018	–	–

Presented are association values (i.e., point-biserial correlation coefficients, bounded between 0 and 1) and their respective p-value (derived from permutations, and corrected for multiple testing) for the 13 compounds that had a significantly higher association with one group.

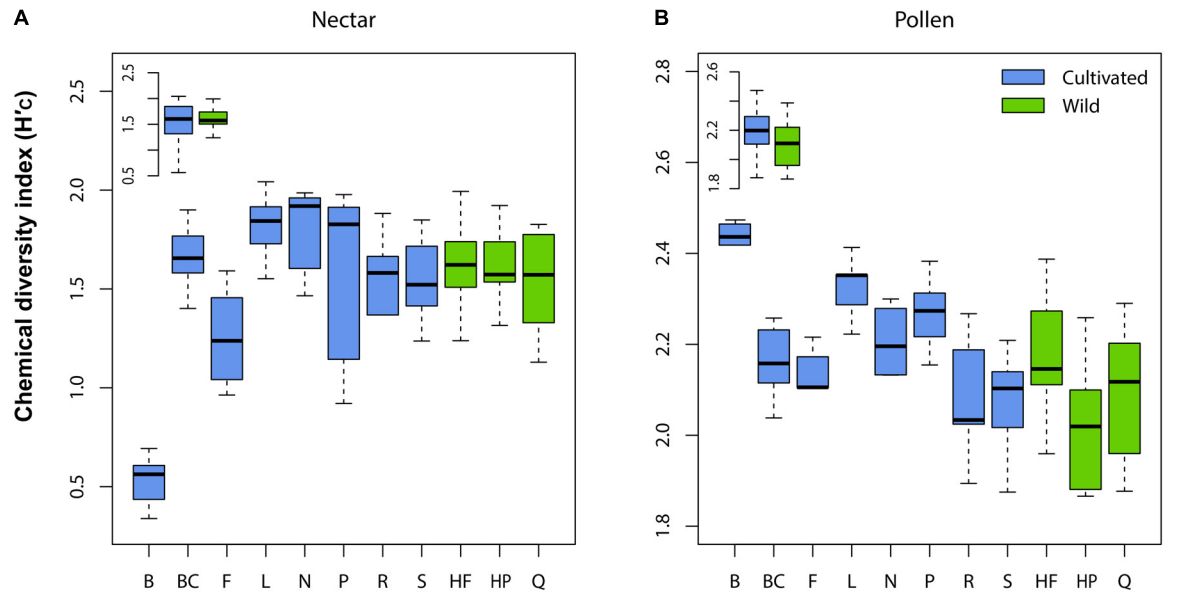

FIGURE 2 | Variation in chemical diversity index (H'_C) across cultivars and wild populations of *Vaccinium corymbosum* for nectar **(A)** and pollen **(B)**. Significant differences between cultivars were observed for both material types, but not between wild populations. No differences in H'_C existed overall between wild and cultivated plants (inset in **A,B**). Abbreviations for cultivars/populations are listed in Methods.

alteration can potentially affect pollinator health via changes in pathogens. While several aspects of nectar and pollen chemistry differed markedly between cultivated and wild plants, this pattern did not hold for all chemical traits, indicating that some traits appeared robust to the domestication process. Of the four hypotheses initially posed, we found at least partial evidence to support each, as discussed in the following sections.

TABLE 2 | Genetic variation, broad-sense heritability, and genetic correlation of nectar and pollen chemistry across six *Vaccinium corymbosum* cultivars ('Bonus,' 'Friendship,' 'Liberty,' 'Northland,' 'Patriot,' 'Reka' – $n = 5$ plants per cultivar, $n = 4$ for 'Northland').

Trait	Material type	Genetic variation (χ^2)	Heritability (H^2)	Genetic correlation					
				Chemical diversity	Chemical composition	Total Amino acids	Total Phenolic acids	Total Flavonols	Chemical diversity
Chemical diversity	Nectar	19.5**	0.70 ± 0.19**	−0.04	0.04	0.05	0.03	0.04	0.01
	Pollen	14.6**	0.63 ± 0.20**						
Chemical composition	Nectar	21.5**	0.73 ± 0.18**	0.41	0.17	0.76†	0.76†	0.86†	0.61†
	Pollen	34.2**	0.85 ± 0.14**						
Total Amino acids	Nectar	22.6**	0.74 ± 0.17**	−0.30	−0.45	−0.11	0.77†	0.89†	0.60†
	Pollen	2.4	0.25 ± 0.18**						
Total Phenolic acids	Nectar	2.1	0.23 ± 0.17*	−0.49	−0.52	0.35	−0.03	0.87†	0.73†
	Pollen	14.0**	0.63 ± 0.20**						
Total Flavonols	Nectar	0.0	0.00 ± 0.09	0.07	0.09	0.004	−0.09	0.06	0.68†
	Pollen	34.7**	0.86 ± 0.13**						
Total Flavan-3-ols	Pollen	7.4*	0.47 ± 0.21**	-	-	-	-	-	-

*Magnitude of genetic variation is indicated by the chi-squared statistic (χ^2). Within-material genetic correlation is given above and below the diagonal for pollen and nectar, respectively, and between-material correlation on the diagonal. Values for total Flavan-3-ols are presented for pollen only, as this compound class was not identified in nectar. †statistical support inferred from Bayesian 95% credible intervals. *p = < 0.05; **p = < 0.001.*

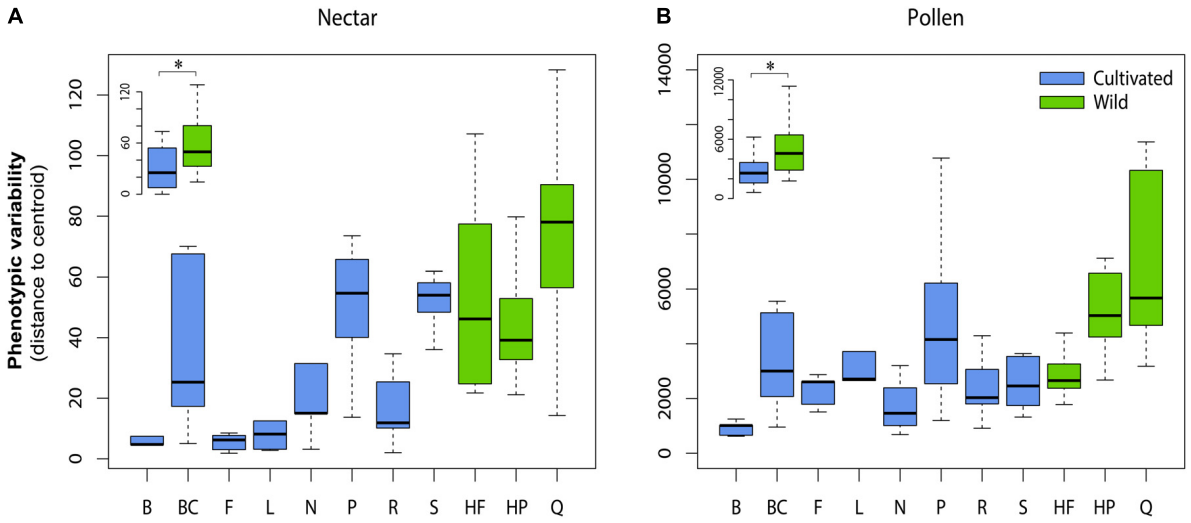

FIGURE 3 | Phenotypic variability in the chemical composition of cultivars and wild populations of *Vaccinium corymbosum* for nectar **(A)** and pollen **(B)**. Phenotypic variability was measured as the Euclidian distance (or dispersion) of plant individuals from their respective cultivar or population multivariate centroid. Significant differences existed overall between wild and cultivated plants for nectar and pollen (inset in **A,B**). Cultivar/population abbreviations are listed in Methods. *$p = < 0.05$.

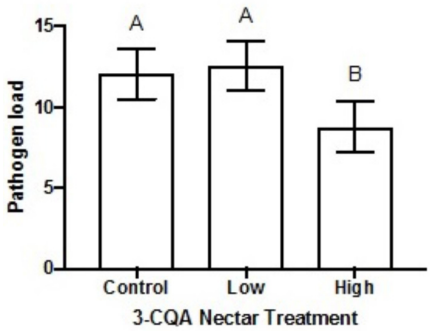

FIGURE 4 | Mean pathogen load of *Crithidia* (log-transformed *Crithidia* per mL + 1) in *Bombus impatiens* workers (± SEM) given different dietary concentrations of 3-*O*-caffeoylquinic acid (3-CQA). The control treatment contained 30% sucrose solution. The low treatment corresponded to 2.8 μM (= 1 ppm) 3-CQA dissolved in 30% sucrose solution, and the high treatment to 56.5 μM (= 20 ppm) dissolved in 30% sucrose solution. Different upper-case letters above bars indicate statistically significant differences at $p < 0.05$.

Domestication Alters Chemical Composition and Diversity of Floral Rewards

As hypothesised, wild and cultivated plants of *V. corymbosum* were differentiated in terms of nectar and pollen chemical composition. However, only pollen had reduced chemical diversity due to domestication; cultivated pollen lacked four flavonoid glycosides which were found in wild pollen. Of the 13 nectar and pollen compounds identified as underlying this differentiation, a majority were positively associated with (or more abundant in) wild plants. However, some examples of the opposite pattern (i.e., greater association with cultivated plants) were also observed for most compound classes (amino acids, phenolic acids, and flavonols), indicating that these differences did not follow a fixed biosynthetic pattern. Flavan-3-ols (catechin and epicatechin, identified only in pollen) did not specifically associate with either group. Of the compounds that differentiated wild or cultivated plants, most did so only for a single material (nectar or pollen). Only two compounds did so for both; phenylalanine showed a consistent pattern across nectar and pollen (positively associated with wild plants for both), whereas the flavonol avicularin showed opposite patterns in nectar and pollen (positive associations with wild plant nectar and cultivated plant pollen).

These results indicate that although domestication effects on the chemical composition and diversity of floral rewards were clear, it is difficult to make *a priori* predictions about which compounds (or compound classes) are affected and in which reward. This difficulty is surprising given the excellent knowledge of the domestication process for highbush blueberry (Hancock and Siefker, 1982; Gough, 1993; Ehlenfeldt, 1994, 2009), in which cultivar provenances, and the characteristics selected for by breeders, are unambiguously known. Selection has typically targeted fruit quality traits such as size, colour, firmness, flavour, and in more recent times, antioxidant content (Kalt et al., 2001). Among various antioxidants, blueberries are a rich source of chlorogenic acid (3-CQA) and anthocyanins, and these compounds correlate with each other in fruit across a wide range of blueberry cultivars (Yousef et al., 2016). Given ongoing selection for rich fruit colour – and by extension 3-CQA – it is reasonable to expect that other caffeic acid esters should increase in floral rewards with domestication. While our results indicated this is the case for 5-CQA in pollen, domestication instead appears to have reduced expression of 4-CSA in nectar. This finding also suggests that fruit chemistry is probably not an accurate proxy for floral reward chemistry. As a whole, given its

general unpredictability, we suggest that floral reward chemistry deserves to be monitored in its own right as part of the crop improvement process for blueberry, and possibly entomophilous crops more generally.

Opportunities and Challenges for Breeding

The majority of nectar and pollen traits examined in cultivars exhibited significant genetic variation and broad-sense heritability, as predicted. The existence of genetic variation in traits is an important prerequisite for selective breeding. In principle, its occurrence offers breeders the potential to fine-tune floral reward chemistry, should such a need be identified. However, genetic correlations (or pleiotropy) may otherwise complicate this process (Luby and Shaw, 2009), and mean that selection cannot be precisely targeted to a trait without causing correlated responses in others, regardless of whether these are desired or not. However, based on two lines of evidence, our findings suggested that different genetic architectures underlie most nectar and pollen chemical traits examined. First, large differences in genetic variation existed for some of the same chemical traits in nectar and pollen; and, second, a majority of within- and between-material genetic correlations were weak or insignificant. Both findings suggest differential genetic regulation of these particular traits, and that pleiotropy does not impose a major constraint. Several pollen chemical traits were exceptions, however, in which large within-material genetic correlations indicate that selection for one trait cannot be achieved independently of others.

Other agronomic metrics of concern to plant breeders include trait and cultivar stability. Here, partitioning phenotypic variance into its total genetic and environmental components allowed us to calculate broad-sense heritability (H^2). H^2 also serves as a relative measure of trait stability; low values indicate that most phenotypic variance is the result of environmental plasticity. Somewhat contrary to the prevailing view of nectar traits as particularly plastic (Mitchell, 2004; Parachnowitsch et al., 2018), nectar H^2 values were mostly high, and generally equivalent to those of pollen. However, total flavonols in nectar were especially unstable (i.e., subject to large environmental variation), as opposed to possessing high stability in pollen. 'Bonus' and 'Friendship' were the most stable cultivars, based on low variability in their multivariate chemical composition of nectar and pollen. These cultivars hence represent good candidates for cultivation, should stability in floral reward chemistry be an important criterion. Interestingly, the effect of interspecific breeding (to produce hybrid or introgressed cultivars) on stability was not consistent. 'Friendship' (a natural wild-collected hybrid between highbush and lowbush blueberry) was one of the most compositionally stable cultivars, whereas 'BlueCrop' and 'Patriot' (which consist of a 6.4 and 28% genetic contribution from lowbush blueberry, respectively (Lobos and Hancock, 2015) – **Supplementary Table 1**) were the least stable.

Although we could not directly assess the genetic merit of the wild populations sampled, these likely represent good sources of genetically diverse germplasm. The cultivated genepool of *V. corymbosum* has not suffered genetic erosion to the same extent as more distantly domesticated crops, although a narrowing in diversity has nonetheless occurred in recent times (Boches et al., 2006). As discussed, we found little to no genetic variation for a minority of nectar and pollen traits examined, which limits their breeding potential. Given this situation, collection of wild germplasm with the aim to enhance genetic variation in floral reward chemistry could be a desirable and feasible prospect.

Impacts of Domestication on Pollinator Health

There was evidence to support our hypothesis that domestication effects on antimicrobial compounds in nectar (i.e., 4-CSA in *V. corymbosum*) could impact pollinator health via changes in pathogens. We found concentration-dependent effects of a nectar secondary compound on the gut pathogen *Crithidia*. Low levels of 3-CQA (as a chemically equivalent proxy for 4-CSA at its typical levels in cultivated plants) had no effects on pathogen counts, while high concentrations (within the natural range for 4-CSA in wild plants) significantly reduced *Crithidia* relative to low and control solutions. Our results extend prior research documenting the *in vivo* medicinal value of some secondary metabolites against *Crithidia* (Manson et al., 2010; Richardson et al., 2015). These effects may reflect the effects of these compounds on the bee immune system rather than direct toxicity to parasite cells, given that up to 2500 ppm 3-CQA had no direct effects on *Crithidia* growth in cell culture (Palmer-Young et al., 2016). Moreover, our results are consistent with other studies showing concentration-dependent effects of plant secondary metabolites on insects and their pathogens (Hunter and Schultz, 1993; Cory and Hoover, 2006; Mao et al., 2013; Palmer-Young et al., 2017). Future work should address whether high dietary consumption of 3-QCA or 4-CSA would benefit bee health via reduced pathogen load in natural settings, and whether there are sublethal costs of consuming caffeic acid esters over short and long time-periods. Nonetheless, utilising wild germplasm to breed new cultivars with enhanced levels or profiles of nectar antimicrobials may hold potential.

Beyond effects on pathogens, other facets of pollinator physiology, ecology, and behaviour are also likely influenced by domestication effects on floral reward chemistry. We observed significantly higher associations of the amino acid phenylalanine with wild plant nectar and pollen. The average concentration of this amino acid in wild plant nectar was nearly 4.5 times that of cultivated plants, and nearly 11 times that of cultivated plants for pollen (**Supplementary Table 2**). Phenylalanine is one of several essential amino acids in bee diets (de Groot et al., 1953). However, arguably its most important function is its strong phagostimulatory quality to bees (Inouye and Waller, 1984; Petanidou et al., 2006), believed to enhance nectar taste (Gardener and Gillman, 2002), and influence bee foraging preferences and behaviour at the community scale (Petanidou, 2007). Reduced phenylalanine levels in the nectar and pollen of cultivated *V. corymbosum* suggest large potential ramifications for pollinator attraction and crop pollination success.

For instance, several highbush cultivars are relatively unattractive to bees (Filmer and Marucci, 1964; Huber, 2016). The reasons for this are unknown, but result in the need for 'saturation pollination' with honey bees (Gough, 1993). A diminished phagostimulatory quality of floral rewards provides one possible explanation for these observations. This hypothesis warrants further investigation.

Future Research Directions

The potential repercussions of crop domestication for pollinators, mediated through floral reward chemistry, have hitherto remained unexamined for animal-pollinated crops. Our findings on highbush blueberry suggest this topic warrants much wider attention for crops more generally. We therefore suggest future research in four complementary areas.

Scale and Trends of Domestication Effects

Establishing the overall scale and specific trends in domestication effects on floral reward chemistry across different types of crop plant would be of great or considerable interest, from both a fundamental and applied perspective. Is alteration of floral reward chemistry a typical outcome of domestication? Does the magnitude of effect depend on the identity of the crop organ(s) artificially selected, as is the case for effects on plant resistance (Whitehead et al., 2017)?

Consequences for Crop Pollinators

While the differential attractiveness of certain crop cultivars to pollinators is a well-known phenomenon (Henning et al., 1992; Klatt et al., 2013; Huber, 2016), to what extent could this be related to altered floral reward chemistry, potentially acting in concert with other floral traits? Conversely, could domestication have served to improve floral rewards, such as by reducing toxic or deterrent compound levels [analogous to the response of nectar toxins to plant invasion (Egan et al., 2016; Tiedeken et al., 2016)], and/or boosting levels of attractive or beneficial ones (e.g., pertaining to nutrition, disease resistance, behaviour, phagostimulation)? Are acute impacts of altered floral reward chemistry readily detectable for pollinators, or are negative effects more likely to be chronic, or only apparent in a context-specific manner such as due to disease (de Roode et al., 2013)?

Mechanistic Understanding of Change

Elucidation of the physiological and genetic mechanisms through which domestication can alter trait function would permit a deeper understanding of this process in crops. For instance, are some compound classes more likely to be affected than others? Does floral reward chemistry usually show independent genetic regulation, or genetic constraints, which may render it less or more robust to alteration than other plant tissues?

Breeding Applications

As has been recognised for other nectar-related traits (Prasifka et al., 2018), tailoring nectar and pollen chemistry for enhanced attraction and functional benefits for pollinators could represent a promising future direction for crop improvement and selective breeding. What are the practical implications for breeding? Can such benefits be unambiguously demonstrated in field settings? What role could wild germplasm play in helping to restore floral rewards which show diminished chemical function, as for other mutualism-related chemical traits (Stenberg et al., 2015)?

Conclusion

As a whole, progress on the above areas would offer great insight into crop domestication effects on floral reward chemistry, and the implications and potential breeding solutions for pollinators and crop pollination.

DATA AVAILABILITY

All datasets and R code used in this study will be made publicly available on Figshare.

AUTHOR CONTRIBUTIONS

LA, RI, PS, and PE conceived and designed the research. IF and PS performed the chemical analyses. PE analysed the data. PE wrote the manuscript with input and contributions from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01357/full#supplementary-material

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Nectar Replaced by Volatile Secretion: A Potential New Role for Nectarless Flowers in a Bee-Pollinated Plant Species

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The presence of nectarless flowers in nectariferous plants is a widespread phenomenon in angiosperms. However, the frequency and distribution of nectarless flowers in natural populations, and the transition from nectariferous to nectarless flowers are poorly known. Variation in nectar production may affect mutualism stability, since energetic resource availability influences pollinators' foraging behavior. Here, we described the spatial and temporal nectar production patterns of *Jacaranda oxyphylla*, a bee-pollinated species that naturally presents nectarless flowers. Additionally, we compared nectariferous and nectarless floral disks in order to identify histological, subcellular and chemical changes that accompanied the loss of nectar production ability. For that we used standard methods for light and transmission electron microscopy, and gas chromatography coupled to mass spectrometry for chemical analyses. We verified that 47% of flowers did not produce nectar during the whole flower lifespan (nectarless flowers). We also observed remarkable inter-plant variation, with individuals having only nectarless flowers, others only nectariferous ones and most of them showing different proportions of both flower types, with variable nectar volumes (3–21 μl). Additionally, among nectariferous flowers, we registered two distinct rhythms of nectar production. 'Early' flowers produced nectar from 0 to 24 h, and 'late' flowers produced nectar from 24 to 48 h of anthesis. Although disks from nectariferous and nectarless flowers displayed similar histological organization, they differed strongly at subcellular level. Nectariferous ('early' and 'late') flowers exhibited a cellular apparatus typical of nectar secretion, while nectarless flowers exhibited osmophoric features. We found three aliphatic and one aromatic compound(s) that were detected in both the headspace of flowers and the disks of nectarless flowers, but not the disks of nectariferous flowers Although the remarkable variation in nectar availability may discourage pollinator visits, nectarless flowers might compensate it by producing volatile compounds that can be

part of floral scent, acting as chemical attractants. Thus, nectarless flowers may be helping to maintain pollination in this scenario of trophic resource supply scarcity. We suggest that *J. oxyphylla* can be transitioning from a nectar-based pollination system to another resource-based or even to a deceit mechanism of pollination.

Keywords: nectar secretion, nectariferous and nectarless flowers, nectary anatomy and ultrastructure, plant–pollinator interactions, volatile compound secretion

INTRODUCTION

The characteristics of floral attractants, including primary ones, such as trophic resources, and secondary ones, such as chemical and visual signals, have strong influence on the establishment of plant–animal interactions (Chittka and Thomson, 2001; Armbruster and Muchhala, 2009; Schaefer and Ruxton, 2011). Floral nectar appeared on the late Cretaceous (Labandeira, 2002) and has since become key trophic resource mediating plant–pollinator interactions (Willmer, 2011). However, spatial and temporal variation in nectar production is commonly described in angiosperms, with differences reported among and within species, plants, and flowers (Pacini and Nepi, 2007; Lu et al., 2015; Zhao et al., 2016). Pollinators can react to variations in nectar features, and the optimal foraging theory, based on caloric consumption, has succeeded to explain their foraging behavior (Pyke, 2010, 2016). So, variation in nectar production may affect mutualism stability by influencing pollinators' foraging behavior (Real, 1981), which may compromise plant reproductive fitness (Zhao et al., 2016). Thus, characterizing how this trophic resource is spatially distributed and how it is temporally released by flowers in a natural population could help to assess the impact of the presence of nectarless flowers on nectarivores' visitation.

Bignoniaceae is known by the presence of zoophilous flowers (Gentry, 1974), with most species presenting nectar as trophic resource, which is produced by a conspicuous nectariferous annular disk that surrounds the ovary base (Galetto, 1995). However, some Bignoniaceae species may present nectarlessness flowers, which has been associated with the absence of a disk (Hauk, 1997), or with the presence of vestigial and non-secretory disks (Rivera, 2000) and with pollination by deceit (Umaña et al., 2011). In spite of *Jacaranda oxyphylla* Cham. being referred as a plant species that possesses a cylindrical nectary disk (Gentry and Morawetz, 1992), around half of its flowers was nectarless in natural populations (Guimarães et al., 2008). However, the causes and consequences of this phenomenon remain unknown. In other plant families, the transitions from nectarless plant species to nectariferous ones has been suggested to rely on subcellular modification, since no morphological differences between nectariferous and nectarless species have been found (Hobbhahn et al., 2013). However, nectary changes related to variation in nectar production within species remains unexplored. Thus, understanding the cellular basis driving the performance of nectariferous and nectarless flowers is essential to explain intra-species nectar variability. Here, we aimed to describe the spatial and temporal variation in nectar production at population level. Additionally, we performed a comparative investigation of the chemical composition and subcellular apparatus of nectariferous and nectarless floral disks in order to identify functional variations that might have accompanied the loss of nectar production ability. Finally, we discussed the potential ecological implications of presenting nectariferous and nectarless flowers focusing on plant–pollinator interactions.

MATERIALS AND METHODS

Study Site and Plant Species

This study was conducted in natural populations of savanna physiognomies "Cerrado" located in Pratânia (22° 48′52″S, 48° 44′35″W) and Botucatu municipalities (22° 57′ 38″S, 48° 31′ 22″W) in São Paulo, Brazil. The field study was performed during the blooming period of the species that occurred at the end of dry season (August–October). This study is part of a long-term project that started in 2006 and is still active, and the data presented here has been collected in the years 2006, 2010–2011, and 2017.

Jacaranda oxyphylla Cham. (Bignoniaceae) varies from sub-shrubby to shrubby habit (**Figure 1A**) and presents branchlets with bipinnate leaves, inflorescences as terminal panicles (**Figure 1B**) bearing flowers with cupular calyx, tubular–campanulate magenta to purplish blue corolla above a narrow basal tube (Gentry and Morawetz, 1992), which corresponds to the nectar chamber (Guimarães et al., 2008). Flowers present didynamous stamens with dithecate anther and a long sub-exerted staminode, a flattened–ovate ovary slightly contracted at the base to a cylindrical disk; elliptic thinly woody fruit with hyaline-membranaceous seeds (Gentry and Morawetz, 1992). Medium-sized bees *Eulaema nigrita* (**Figure 1C**) and *Bombus morio*, small-sized bee *Exomalopsis fulvofasciata* and, occasionally, hummingbirds visited the flowers in a legitimate way and behaved as pollinators; while *Oxaea flavescens* (**Figure 1D**) and *Xylocopa* sp. acted as nectar robbers (Guimarães et al., 2008).

Vouchers are deposited in the 'Irina Delanova Gemtchujnicov' Herbarium (BOTU) of the Biosciences Institute of the São Paulo State University (UNESP), Botucatu, Brazil, under numbers 24408–24412.

Nectar Production Variability

For the nectar sampling described in subsections "Characterizing Nectar Production in Space: Variation Within and Among Plants" and "Characterizing Nectar Production in Time: Variation Throughout Anthesis," we maintained all the sampled flowers isolated with bridal veil bags, since bud stage, in order to prevent nectar withdrawn by floral visitors, as recommended

FIGURE 1 | *Jacaranda oxyphylla* (Bignoniaceae) and its floral visitors. **(A)** Study site showing "Cerrado" vegetation and four individuals of *J. oxyphylla* (arrows). Scale bar: 13 cm; **(B)** detail of an inflorescence of *J. oxyphylla*. Scale bar: 5 cm; **(C)** *Eulaema nigrita*, one of the pollinators of *J. oxyphylla*, visiting a flower. Scale bar: 1.5 cm; **(D)** *Oxaea flavescens*, the main nectar robber of *J. oxyphylla* flowers, visiting a flower. Scale bar: 0.5 cm.

by Corbet (2003). The nectar volume was always measured using graded glass syringes (10 μl).

Characterizing Nectar Production in Space: Variation Within and Among Plants

In order to characterize floral nectar availability in space, considering both within and among plants variation in nectar production, we described the spatial distribution of *J. oxyphylla*, by measuring the Cartesian distances among plants in a natural population (15 plots of 100 m^2, totaling 1,500 m^2) and calculating Morisita's dispersion index (Morisita, 1959, 1962). To characterize the variation in the amount of nectar potentially available to pollinators, we sampled all the 48 h and 72 h flowers in 29 plants (totaling 205 flowers, 7 ± 4 flowers per plant).

Then, we determined the percentage of nectarless flowers in our study population and we evaluated the variation of nectar production within plants by determining the proportion of nectariferous and nectarless flowers per plant. Additionally, we compared the frequency of nectariferous and nectarless flowers among plants using Pearson's Chi-squared test, in order to verify if the plants showed distinct proportions of both types of flowers. Also, we evaluated if the mean volume of nectar produced by flowers varied among plants, which allowed us to identify if individual plants had an influence on nectar volume production. For this, we used ANOVA with Brown–Forsythe correction for heteroskedastic data and Games–Howell *post hoc* test for pairwise comparisons. Additionally, we counted the number of inflorescences per plant (n = 40 plants) and the number of flowers per inflorescence (n = 46 inflorescences, 40 plants). We verified if there was any influence of flower position within inflorescences on the accumulated nectar volume by using a regression analysis (n = 35 inflorescences, 18 plants).

We also verified the probability of finding nectarless flowers in the apex and the base of inflorescences (n = 22 inflorescences, 14 plants), using generalized linear model (GLM) with Binomial error distribution.

Characterizing Nectar Production in Time: Variation Throughout Anthesis

We determined whether nectar accumulation started in bud stage by inspecting pre-anthesis floral buds (n = 30 buds from 20 plants). In order to verify if nectarless flowers have no nectar during their whole lifespan or if they were actually product of nectar resorption, in addition to characterize the nectar secretion pattern, we described the daily nectar secretion (with removal) and the accumulated nectar production throughout anthesis. To evaluate nectar secretion with removal during the whole flower lifespan, we sampled flowers at intervals of 24 h, starting at the moment of flower opening in the first day of anthesis (0 h) and ending at the seventh day of anthesis, totaling 144 h of monitoring. We finished our sampling at the seventh day because, at that moment, only 15% of the flowers were still attached to the inflorescences. For that, we used a set of 45 bagged flowers (n = 20 plants, 1–3 flowers per plant). Every 24 h, we removed each individual bag, withdrew all the nectar from each flower, and immediately bagged it again. We resampled the same flowers every 24 h until corolla abscission or until 144 h of anthesis. Then, in order to identify if the daily nectar production (with removal) differed among days of anthesis, we compared the volumes of nectar produced at each day, using ANOVA with Brown–Forsythe correction for heteroskedastic data and Games–Howell *post hoc* test for pairwise comparisons. We also performed Local Weighted Regression (LOESS) with 95% confidence intervals to describe the variation in daily nectar production throughout the anthesis. To determine the accumulated nectar volume, we performed a set of experiments in which we sampled the nectar in flowers at every 24 h from 0 h to 48 h of anthesis (n = 81 flowers from 39 plants, 1–3 flowers sampled per plant). However, instead of resampling the same flowers at every interval, we sampled the accumulated nectar volume in a different set of flowers each time. So that, each set of flowers was sampled just once and then discarded. Around 0700 h (time of flower opening), in the first day of anthesis, we sampled nectar from a set of 30 flowers (0-h flowers). In sequence, 28 different flowers were sampled at 0700 h in the second day of anthesis (24-h flowers), and 23 flowers in the third day of anthesis (48-h flowers). We also verified if the volume of accumulated nectar varied among days of anthesis using Kruskal–Wallis test, and performed LOESS with 95% confidence intervals to describe the secretion pattern in flowers with accumulated nectar. Additionally, we performed Wilcoxon Rank Sum test to verify if there were any differences between the sum of the daily nectar production (with removal) and the accumulated nectar (during the part of anthesis in which there was nectar production) in order to investigate if there was any effect of nectar removal on secretion pattern. The sum of the daily nectar production at 24 h corresponds to the volume withdrawn at 0 h + 24 h, and the sum of the daily nectar production at 48 h corresponds to the volume withdrawn at 0 h + 24 h + 48 h.

Thirty-eight flowers used in the accumulated nectar experiment were nectarless. The remaining nectariferous flowers (n = 43 from 30 plants, 1–3 flowers per plant) were used to determine total concentration of nectar (% w/w) along the three time intervals (0 h, 24 h, 48 h) by mean of a hand-held refractometer. We used both nectar volume and concentration parameters to estimate the total milligrams of sugar produced per flower, as proposed by Galetto and Bernardello (2005). Then, we compared nectar concentration and total milligrams of sugar (mgS) per flower throughout anthesis using ANOVA with Brown–Forsythe correction for heteroskedastic data and one-way ANOVA, respectively. All the statistical analyses were performed in R v. 3.3.1 (R Development Core Team, 2016) and in R v. 3.4.3 (R Development Core Team, 2018) with standard and additional packages: ggplot2 (Wickham, 2009), msir (Scrucca, 2011), and userfriendlyscience (Peters, 2017).

Histological and Cellular Analyses

We performed Kruskal–Wallis rank sum test and we found that nectariferous and nectarless flowers did not differ in their longevity [$X^2_{(1)} = 0.0571$, $p = 0811$]. We sampled flowers of both types based on the periods of nectar secretion of nectariferous flowers, from 0 until 48 h of anthesis. We also sampled disks after nectar production cessation (72 h of anthesis). We also compared the disk volume of nectariferous and nectarless flowers by measuring the height and the diameter with a digital caliper (Mytutoyo®, United States) in 13 flowers, from eight plants.

For histological characterization of the disks, we fixed disk samples (n = 10 for each flower type) in Karnovsky's solution (4% paraformaldehyde; 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2; 0.2 M phosphate buffer, pH 7.2) for 24 h (Karnovsky, 1965), and we dehydrated them in an ethanol series (50, 70, 90, 100%) and embedded them in methacrylate resin (Historesin®, Leica, Wetzlar, Germany) in accordance with the manufacturer's recommended procedure. We obtained the sections (4–6 μm) using a Leica RM2255 rotary microtome and we stained them with 0.05% toluidine blue, pH 4.5 (O'Brien et al., 1964). We carried out histochemical tests on material fixed in Karnovsky solution, both in sections obtained by free hand and by microtome after inclusion in resin. We applied the following histochemical tests: 10% aqueous ferric chloride solution for phenolic compounds identification (Johansen, 1940); Lugol for the identification of starch (Johansen, 1940); Sudan IV for lipids in general (Johansen, 1940), Sudan Black B for lipids in raw nectar, as described by Kram et al. (2008); and NADI's reagent (α-naphtol and *N*,*N*-dimethyl-*p*-phenylenediamine) for the detection of resin or essential oils (David and Carde, 1964). The presence of phenolic substances was checked by staining with toluidine blue, according to Ramalingam and Ravindranath (1970). We analyzed the slides under a Leica DMR microscope with image capture system (Leica DFC 425).

For ultrastructural analyses, we fixed disk fragments in glutaraldehyde (2.5% with 0.1 M phosphate buffer, pH 7.3, for 6–8 h at 4°C) and post-fixed them with osmium tetroxide (1% in the same buffer, for 2 h at room temperature). After a washing in distilled water, we stained the materials with 0.5% uranyl acetate in water solution for 2 h at room temperature. Afterward,

we dehydrated the samples in a graded acetone series (50, 70, 90, and 100%), and embedded them in Araldite® resin at room temperature. We carried out the polymerization at 60°C for 48 h and stained the semi-thin sections with 1% toluidine blue, while ultra-thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). We observed the sections under transmission electron microscopy (TEM), Tecnai Spirit (FEI) microscope, at 80 kV.

Chemical Analysis

To verify if the disks of both nectariferous and nectarless flowers contain volatiles that are also found in the headspace of flowers, we compared the volatile compounds present in the disks with the floral scent of *J. oxyphylla*.

To evaluate the chemical compounds present in the disks, we collected disks from 15 nectariferous and 18 nectarless flowers (n = 5 plants for both types of flowers), and separated them from the flowers using razor blades. All of the flowers were bagged since bud stage and were collected at approximately 48 h of anthesis. Disk samples were stored in a freezer at approximately −80°C. Later, we analyzed the samples on a Thermo Scientific GC-MS, model FOCUS equipped with an automatic sampler (Thermo – triplus DUO) and coupled to a Thermo – ISQ 230ST mass detector. We used a TG-5MS column for the analysis (30 m long, 0.25 mm of inner diameter, 0.25 μm of film thickness) and we maintained a constant 1 mL/min flow of helium as the carrier gas. The disks were accommodated in vials at 200°C for 15 min in a heating stove prior to the injection. An automated gas tight syringe was maintained at 140°C, perforated the vial seal, collected 2 mL of sample from inside the vial and immediately injected the sample into the GC injector in splitless mode, with the injector temperature being 200°C. The samples were co-injected with a 500 μL mixture of n-alkanes (C7–C30) at 0.1% of concentration that was added to the vials containing the disks. Column temperature was initially 50°C, then increased by 5°C/min to 250°C and kept constant for 5 min. The MS interface was at 250°C. The detector was operated in electron impact ionization mode (70 eV), with a scanning range of 34–350 m/z. Given that cutting and heating of the disks will produce compounds not normally released in the headspace of the flowers, we only looked for compounds detected in *in situ* samples of floral scents. To obtain such samples, we sampled floral scent by dynamic headspace, following the protocol by Dötterl et al. (2005). The sampled flowers (n = 5 flowers from five plants at 0 h of anthesis) were enclosed for 10 min in polyethylene bags (8 × 10 cm). As only recently opened flowers were used for the analyses, it was not possible to effectively determine in the field if these flowers were nectariferous or nectarless, as we found that some nectariferous flowers only start nectar accumulation at 24 h ('late' flowers, see Results section). The volatile compounds which accumulated inside the bags were collected with adsorbent traps connected to a membrane pump, with an air flow of 200 mL/min during 50 min. We used adsorbent tubes with approximately 15 mm of length by 2 mm of internal diameter that were filled with a mixture of 1.5 mg Tenax-TA (60–80 mesh) and 1.5 mg of Carbotrap B (20–40 mesh; both Supelco®). Besides collecting volatile compounds directly from the flowers, we also collected samples from leaves in order to discriminate any possible contaminants or not flower-exclusive volatile compounds. Samples were stored in a freezer at approximately −80°C. We analyzed the samples on an automated thermo desorption system (Model TD-20; Shimadzu, Kyoto, Japan) coupled to a GC-MS (model QP2010 Ultra EI; Shimadzu) equipped with a ZB-5 fused silica column (60 m long, 0.25 mm of inner diameter, 0.25 μm of film thickness), as described in Mitchell et al. (2015). We maintained a constant 1.5 mL/min flow of helium as the carrier gas. The injector temperature was 200°C. Oven temperature started at 40°C, then increased by 6°C/min to 250°C and kept constant for 1 min. The MS interface was at 250°C. Mass spectra were taken at electron energy 70 eV (in EI mode), with scanning range of 30–350 m/z.

In all of the analysis, we carried out tentative compound identification using NIST 08, and Adams (2007) mass spectral libraries. Final identification was carried out by comparing the mass spectra and Kovats Retention Indices (RI) of target compounds with that of authentic standard compounds. For quantitative analysis of VOCs, 100 ng each of ca. 150 components, among them monoterpenes, aliphatic, and aromatic compounds, were injected into the GC-MS system. The mean of the peak areas (total ion current) of these compounds was used to estimate the total amount of scent available in the scent samples (Etl et al., 2016).

RESULTS

Nectar Production Variability

Characterizing Nectar Production in Space: Variation Within and Among Plants

We observed that *J. oxyphylla* presents a clumped spatial distribution (I_σ = 0.5013, 95% IC) (**Figure 2**). We noticed that only 12.5% of *J. oxyphylla* individuals presented more than one inflorescence (2–3), and the plants presented a mean of 6.1 ± 3.9 open flowers per inflorescences per day. We found 47% of nectarless flowers in the study population. The frequency of nectariferous and nectarless flowers differed among plants [$X^2_{(30)}$ = 94.49, $P < 0.001$], with 10% of plants showing only nectariferous flowers, 6.5% of plants showing only nectarless flowers and the remaining plants showing variable mixed proportions of both flower types (**Figure 2**). So, we observed a significant influence of plant on nectar volume production ($F_{28,55.98}$ = 4.2, $P < 0.001$) (**Figure 3**). Also, nectar volume was quite variable among nectariferous flowers within each plant (3–21 μL) (**Figure 3**). There was no association between flowers position in the inflorescence and the accumulated nectar volume per flower (R^2 = −0.0285, P = 0.9758). Additionally, the probability of finding nectarless flowers was similar when comparing the basis and the apex of the inflorescences (Z = 0.096, P = 0.924).

Characterizing Nectar Production in Time: Variation Throughout Anthesis

No nectar was found in pre-anthesis bud stage (1 day before anthesis), and nectar presence was registered only at the moment

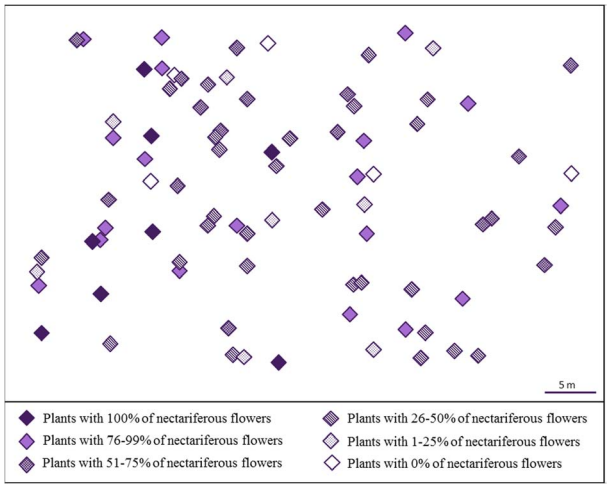

FIGURE 2 | Spatial distribution of *J. oxyphylla* (Bignoniaceae), Botucatu, Brazil. Each diamond represents a single plant. The colors and patterns inside the diamonds indicate the percentage of nectariferous flowers found in each plant.

of flower opening or later. We observed more nectarless flowers in the first day of anthesis than in the following day (**Figure 4A**). Actually, the majority of these first-day nectarless flowers remained nectarless throughout their lifespan. However, part of the flowers that showed no nectar during their first day of anthesis, started producing nectar later on. Considering all the sampled nectariferous flowers, we observed a variation in the daily rate of nectar production per flower during anthesis ($F_{6,88.85} = 9.03$, $P < 0.001$) (**Figures 4A,B**), with a mean production rate of 1.9 ± 3.34 μl in the first day, 1.58 ± 2.66 μl in the second day and 0.83 ± 2.05 μl in the third day, after which nectar production ceased completely. Based on the analysis of the daily nectar production, two groups of flowers were distinguishable in the sampled population when taking into account the beginning of nectar release. In 58% of nectariferous flowers, nectar release started just before flower opening (**Figure 4C**, from now on named 'early' flowers). In these flowers, the maximum volume of nectar occurred at 0 h, followed by the addition of smaller amounts of nectar until 24 h and by production cessation ($F_{6,59.81} = 9.4$, $P < 0.001$) (**Figure 4D**). In the other 42% of nectariferous flowers, nectar release started only by the end of the first day of anthesis (**Figure 4E**, from now on named 'late' flowers), with the maximum volume of nectar registered at 24 h, followed by a smaller production until 48 h and by production cessation ($F_{6,15.1} = 25.66$, $P < 0.001$) (**Figure 4F**). It is noteworthy that, regardless of the day that production started, each flower released nectar during the maximum of 2 days (**Figures 4C,E**). After the discovery that *J. oxyphylla* presented these two distinct nectar production rhythms, we verified if 'early' and 'late' flowers produced similar volumes of nectar during their first day of nectar production (0 h for 'early' flowers and 24 h for 'late' ones), and during their second day of production (24 h for 'early' flowers and 48 h for 'late' ones). For that, we performed Wilcoxon Rank Sum test with continuity correction for unbalanced samples. We observed that the volumes of nectar produced by both flower types were similar in the first and second days of nectar production ($W = 86$, $P = 0.8612$, and $W = 107.5$, $P = 0.2562$, for first and second days of production, respectively).

When we compared the initial overall volume of nectar produced (0 h) to the subsequent volumes produced by flowers (based on the sum of the daily nectar production rates), we observed that in the first day occurred the highest nectar production (from 0 h to 24 h), followed by an insignificant addition of nectar in the subsequent day (from 24 to 48 h), so that the sum of the volumes at 48 h was similar to the sum of the volumes at 24 h of anthesis [$X^2_{(2)} = 6.93$, $P = 0.031$] (**Figure 5A**). Even though we found a high variation in nectar volume when comparing flowers and plants, the overall accumulated amount of nectar per day was similar throughout the first 48 h of anthesis [$X^2_{(2)} = 5.37$, $P = 0.068$] (**Figure 5B**). Additionally, nectar mean concentration (25.91 ± 6.32% w/w) and the total milligrams of sugar per flower (1.35 ± 1.02 mg S) remained constant throughout this period ($F_{3,89.42} = 1.91$, $P = 0.162$; $F_{3,39} = 1.34$, $P = 0.277$, respectively). Finally, we did not observe any difference when comparing the accumulated nectar at 48 h of anthesis with the sum of the daily nectar production from 0 to 48 h ($W = 456.5$, $P = 0.406$).

Comparative Histological and Cellular Analyses

Nectariferous and Nectarless Disks' Histology and Histochemistry

The disk volume in nectariferous flowers (5.50 ± 2.25 mm^3) and in nectarless flowers (5.60 ± 2.11 mm^3) was similar ($t_{14.24} = -0.0998$, $P = 0.922$). Disks from both nectariferous (**Figures 6A–C**) and nectarless flowers (**Figures 6D–G**) at 0 h of anthesis, in cross sections through the median region, were constituted by uniseriate epidermis with stomata (**Figure 6B,E**) across the entire disk surface and several layers of parenchyma vascularized with only phloem (**Figures 6C,F**). Although both disks exhibited a similar histological organization, nectariferous flowers showed more clearly two parenchyma regions, nectary and subnectary parenchyma.

In nectariferous flowers, the nectary parenchyma (underlying the epidermis) was composed by several layers of small, isodiametric, thin-walled cells, with relatively large nucleus, dense cytoplasm, and developed vacuoles (**Figures 6A,B**). The subnectary parenchyma, in continuity with the nectary parenchyma, was composed by larger cells, with irregular shapes, less dense cytoplasm, and larger intercellular spaces (**Figure 6A**). Phloem strands coming from the collateral bundles ramify into the subnectary parenchyma (**Figure 6C**). Parenchyma cells in division were commonly observed in both nectariferous and nectarless flowers (**Figures 6B,E**).

In nectarless flowers (**Figures 6D–G**), the epidermal cells had irregular sizes and shapes and numerous globules in the protoplast (**Figure 6E**). Larger, vacuolated and irregularly shaped parenchyma cells, located just under the stomata, expanded toward the substomatic chamber and kept

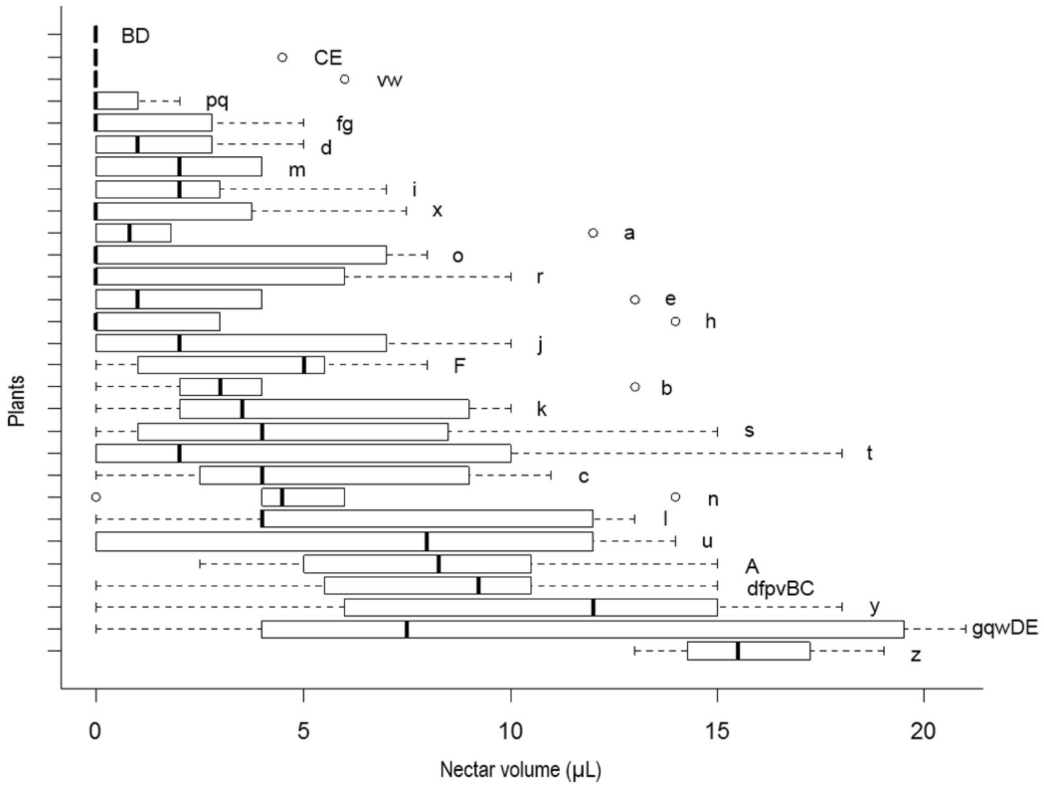

FIGURE 3 | Box plots of nectar volume variability per plant in *J. oxyphylla*. The box plots show the median (vertical line across the box), 25th and 75th percentiles (lower and upper edges of the box) and the upper and lower whiskers, which correspond to the higher and lower data that is no further from the box than 1.5 times the interquartile range. Any data that lied beyond the whiskers was considered an outlier (empty circles). Nectar volumes significantly different from one plant to another are denoted by different letters on the right side of the boxes (ANOVA with Brown–Forsythe correction for heteroskedastic data and Games–Howell *post hoc* test). Nectar volume showed high variability within and among plants ($p < 0.05$). Note that various plants presented flowers with no nectar and others presented outliers as well.

interspersed among the epidermal cells (**Figure 6F**). Stomata with enlarged aperture, and loose or detached epidermal cells were commonly observed (**Figure 6F**). Comparing with nectariferous flowers, the parenchyma region presented lower number of layers composed by juxtaposed cells (**Figures 6D,E**), with small intercellular spaces, vascularized with phloem strands (**Figure 6G**).

Starch grains, phenolic substances, lipid bodies, and essences were detected in both nectariferous and nectarless 48 h flowers. A clear decrease in the amount of starch grains (**Figures 7A–C**) occurred at the begging of anthesis of nectariferous and nectarless flowers (0–48 h). On the other hand, phenolic substances (**Figure 7D**), lipid bodies (**Figure 7E**), and essences (**Figure 7F**) became more abundant throughout anthesis in both flower types. Sudan Black B reacted positively for lipids in nectar (**Figure 7G**). The results of the histochemical tests on the secretory disk of nectariferous and nectarless flowers of *Jacaranda oxyphylla* are summarized in the **Table 1**.

Nectariferous and Nectarless Disks' Ultrastructure

We investigated the subcellular organization of nectariferous and nectarless flowers in *J. oxyphylla* with emphasis on plastid changes, considering the flower life stages in which we observed the presence of nectar (0–48 h of anthesis) and after nectar production cessation (72 h of anthesis).

Nectariferous flowers

'Early' flowers: flowers that started releasing nectar at 0 h of anthesis. Nectary disk from recently opened flowers, at 0 h of anthesis, showed rectangular epidermal cells with voluminous nuclei, dense cytoplasm and little-developed vacuoles containing osmiophilic bodies, flocculent material, oil drops, and membrane debris (**Figure 8A**). Plasmodesmata connected epidermal cells with each other and with the underlying parenchyma (**Figure 8B**). The outer tangential walls were thick, sinuous and covered with a thin, smooth cuticle (**Figure 8B**), which was composed of an inner reticulate layer containing microchannels and an outer amorphous layer that corresponded to the cuticle proper; osmiophilic deposits occurred in the cuticle layer and oil inclusions in the cell wall matrix (**Figure 8C**). The cuticle was continuous and did not have cracks, tears, or pores (**Figures 8B,C**). Large nuclei, free ribosomes, rough endoplasmic reticulum (RER), mitochondria and plastids characterized the epidermal cells in this stage (**Figure 8B**). Plastids, with residual starch grains (**Figure 8A**) or lacking starch (**Figure 8B**) had very electron-dense, homogenous stroma due to phenolic substances

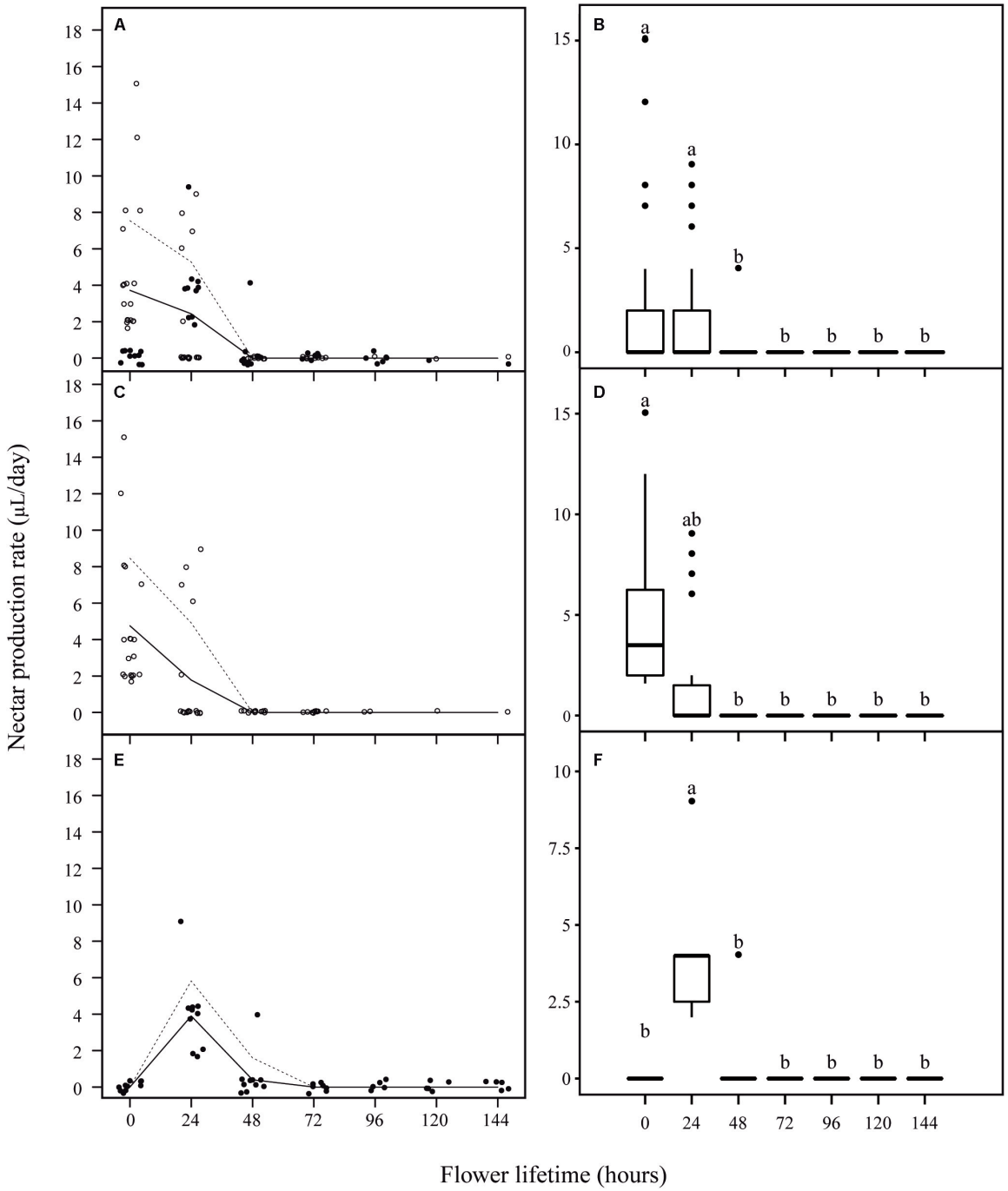

FIGURE 4 | Nectar production rate per flower lifetime in *J. oxyphylla* flowers. In **(A,C,E)**, the trendlines describe a visual relationship between the two variables (nectar production rate and flower lifetime) based on the lowest smoother using a locally weighted regression (LOESS). Dashed lines are 95% confidence interval upper limits. The 95% confidence interval lower limits were zero and the line was omitted. The empty circles represent 'early' flowers and the full circles represent 'late' flowers. In **(B,D,F)**, the box plots show the median (horizontal line across the box), 25th and 75th percentiles (lower and upper edges of the box) and the upper and lower whiskers, which correspond to the higher and lower data that is no further from the box than 1.5 times the interquartile range. Any data that lied beyond the whiskers was considered an outlier (filled circles). Nectar production rates significantly different from one period to another are denoted by different letters above the boxes (ANOVA with Brown–Forsythe correction for heteroskedastic data and Games–Howell *post hoc* test). **(A)** Nectar production rate per flower at every 24 h of anthesis. **(B)** Nectar production rate was similar during the first 2 days of anthesis, followed by an undermost production during the third day of anthesis and ceasing before 72 h of anthesis ($p < 0.05$); **(C)** nectar production rate per 'early' flowers at every 24 h of anthesis; **(D)** in 'early' flowers, the maximum volume of nectar occurred at 0 h, followed by the addition of smaller amounts of nectar until 24 h and by production cessation ($p < 0.05$); **(E)** nectar production rate per 'late' flowers at every 24 h of anthesis; **(F)** in 'late' flowers, the maximum volume of nectar registered at 24 h, followed by a smaller production until 48 h and by production cessation ($p < 0.05$).

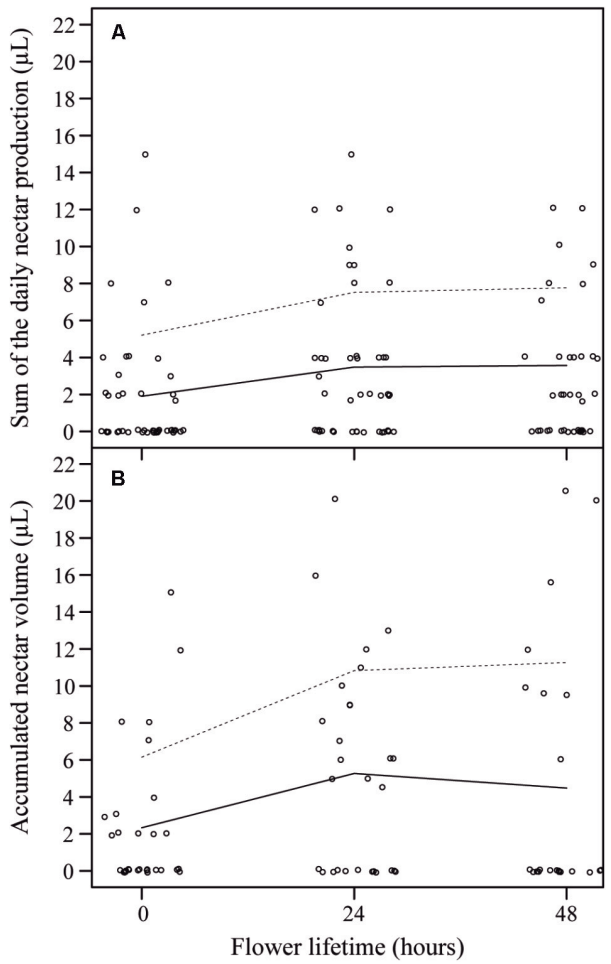

FIGURE 5 | Sum of the daily nectar production per flower lifetime and accumulated nectar volume per flower lifetime in *J. oxyphylla* flowers. **(A)** Sum of the daily nectar production per flower lifetime. The trend lines describe a visual relationship between the two variables (nectar production rate and flower lifetime) based on the lowest smoother using a locally weighted regression (LOESS). Dashed lines are 95% confidence interval upper limits. The 95% confidence interval lower limits were zero and the line was omitted. **(B)** Accumulated nectar volume per flower lifetime. The trend lines also describe a visual relationship between the two variables (nectar production rate and flower lifetime) based on LOESS regression. There were no differences between the sum of the daily nectar production from 0 to 48 h and the accumulated nectar at 48 h of anthesis.

accumulations. Oil drops occurred inside vacuoles (**Figure 8A**), close to the plasma membrane or juxtaposed to the tonoplast (**Figure 8B**). At the same stage, sections of the central region of the disk showed epidermal cells with greater development of vacuoles, sinuous plasmalemma and periplasmic spaces (**Figure 8D**), besides cytoplasm with more evident organelles, especially RER, mitochondria and Golgi bodies (**Figure 8E**). The RER profiles were extensive, exhibited dilated regions and were generally situated in the peripheral cytoplasm, adjacent to the plasma membrane (**Figure 8E**). There were many vesicles near the swollen edges of the RER and their location and arrangement suggested that they had budded off from the RER profiles. Moreover, images suggested the fusion of coated vesicles with the plasma membrane, which showed sinuous contour (**Figure 8E**). Accumulations of flocculent material occurred inside vacuoles (**Figure 8D**) and in periplasmic spaces (**Figure 8E**). Oil bodies occurred scattered in the cytosol, near the plasmalemma or tonoplast (**Figure 8F**). In the same section of the disk, nectary parenchyma cells located side by side exhibited different ultrastructure (**Figure 8G**). In some cells, amyloplasts exhibited reduced starch grains with hydrolysis signals, or residues of starch grains (**Figure 8G**, top right corner). Some cells had small nucleus, numerous undifferentiated vacuoles and dense cytoplasm (**Figure 8G**) with mitochondria and RER profiles, which were more commonly located near the degenerating plastids or surrounding the vacuoles containing flocculent materials (**Figure 8H**). Other neighboring cells showed conspicuous nucleus with evident nucleolus, denser and abundant cytoplasm and merged vacuoles (**Figure 8G**). Amyloplasts were absent in these cells. Flocculent material, probably originated from starch hydrolysis, was incorporated into the cytoplasmic matrix (**Figures 8G,I**). The vacuoles had irregular sizes and shapes and progressively merged with each other forming larger vacuoles (**Figures 8G,I**). Increase in cytoplasmic density and in the amount of mitochondria and oil bodies, besides the appearance of smooth endoplasmic reticulum (SER) with dilated elements characterized these cells (**Figures 8G,I**). SER elements occurred mainly located in the peripheral cytoplasm (**Figure 8I**).

At 24 h of anthesis, most of the nectary parenchyma cells exhibited a similar pattern to the observed at the previous stage, characterized by vacuoles containing flocculent materials and few residual starch grains (**Figure 9A**). Moreover, these cells exhibited conspicuous nucleus with evident nucleolus and dense cytoplasm (**Figure 9A**) rich in polyribosomes, mitochondria, Golgi bodies and extensive RER together vesicles located in the peripheral cytoplasm (**Figure 9B**). The plasmalemma was sinuous in outline and periplasmic spaces contained flocculent materials (**Figure 9B**). At this stage, the occurrence of a distinct plastid type, not observed at previous stages, was remarkable. It featured an elongated shape, granular stroma with small lipid droplets and an irregular, poorly developed inner membrane system (**Figure 9C**). The presence of a narrow constriction in their middle region is noticeable and is an evidence of plastid division. Mitochondria and RER profiles were common around these plastids (**Figure 9C**).

In the sub-nectary region, the parenchyma cells associated or not with phloem, were characterized by a developed vacuole system (**Figure 9D**). Parenchyma cells in this nectary region exhibited numerous undifferentiated chloroplasts through the cytosol, and some of them contained small starch grains and few developed thylakoids (**Figure 9E**). Phloem parenchyma cells in this region also had undifferentiated chloroplasts with thylakoid-like membranes, small vacuoles and abundant SER elements situated in the periphery of the cytoplasm (**Figure 9F**).

At 48 h of anthesis, nectary parenchyma region had more developed intercellular spaces (**Figure 10A**) when compared to the previous stage. In this stage, there was a remarkable occurrence of polymorphic plastids (**Figures 10A,B**)

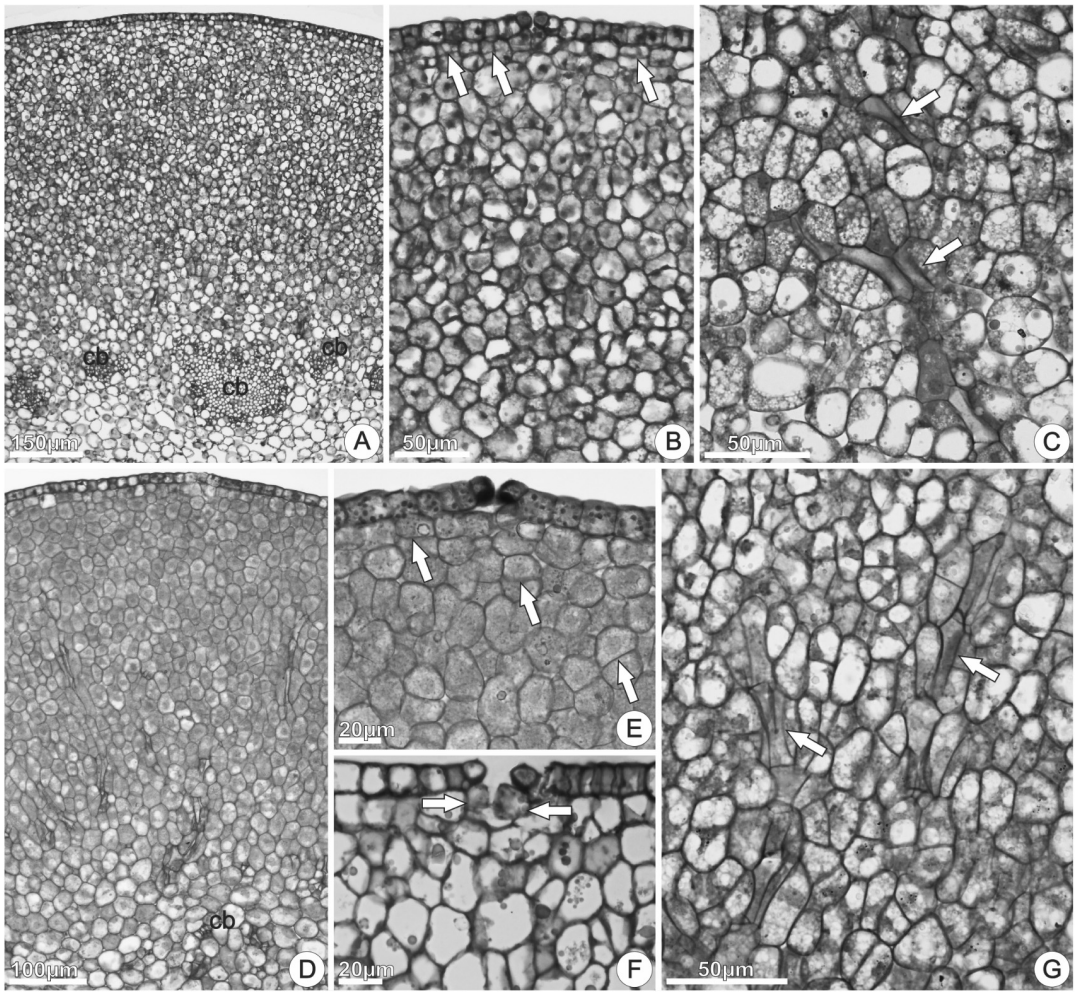

FIGURE 6 | Structure of the floral disk of *Jacaranda oxyphylla,* illustrated by cross **(A,B,D,E,G)** and longitudinal **(C,F)** sections. **(A–C)** Nectariferous flowers; **(D–G)** nectarless flowers. **(A)** General aspect of the disk showing the epidermis, nectary parenchyma, sub-nectary parenchyma and collateral bundles; **(B)** detail of **(A)** showing epidermis coated with thin cuticle, stomata, and nectary parenchyma region composed by isodiametric cells. Arrows indicate cell division in the subepidermal layers; **(C)** subnectary parenchyma with phloem strands (arrows); **(D)** general aspect of the disk showing the epidermis, parenchyma, and vascular tissues; **(E)** epidermal cells with dense globules and stomata with associated secretion. Arrows indicate cell division in the subepidermal layers; **(F)** detached epidermal cells and large parenchyma cells inside substomatic chamber (arrows); **(G)** phloem strands (arrows) in the parenchyma tissue. cb, collateral bundles.

featured by electron-dense stroma, small oil globules and vesicle/tubular inner membranes. The richness in free ribosomes, large mitochondria, SER and RER profiles was noticeable (**Figures 10B–F**), in addition to the considerable increase in number and size of lipid bodies in the cytoplasm (**Figures 10C–E**). In the region of intercellular spaces, cells had organelles located in parietal position; dense granulations occurred adhered to the cell walls, bordering the intercellular space (**Figure 10F**). Across the entire cell surface occurred multivesicular bodies and dilated profiles of RER near the plasmalemma, besides periplasmic space, which was prominent and contained multilamellar membranes and dense granulations (**Figure 10G**). We observed oil drops close to the plastids (**Figure 10B**), scattered (**Figures 10C,D**) or clustered (**Figure 10H**) in the cytoplasm, close to the plasmalemma (**Figure 10E**), and inside vacuoles (**Figure 10I**), where they merged together forming conspicuous oil bodies. At 72 h of anthesis, parenchyma cells had similar features (not shown here).

'Late' flowers: flowers that started releasing nectar at 24 h of anthesis. At 0 h of anthesis, disks from 'late' flowers presented similar features to those from 'early' flowers at 0 h of anthesis, showing abundance of amyloplasts and beginning of starch grains hydrolysis.

At 24 h of anthesis, nectaries disks from 'late' flowers presented similar features as to those from 'early' flowers at 24 h of anthesis showing total or partial depletion of starch grains.

At 48 h, the nectaries from 'late' flowers presented similar features as those from 'early' flowers at 48 h of anthesis, with total depletion of starch grains and incorporation of the amyloplasts residues into the cytoplasmic matrix, changes in plastid type and an increase of lipid inclusions.

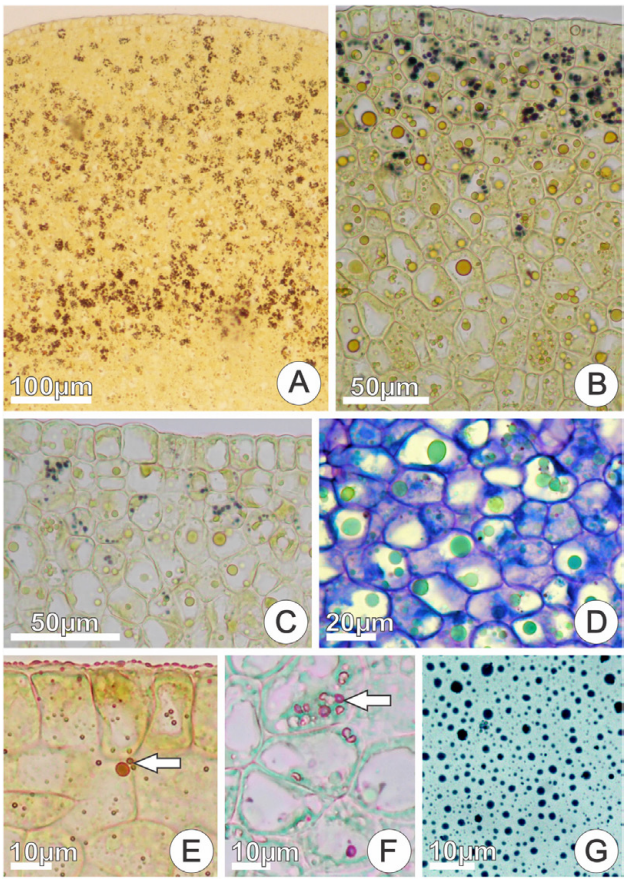

FIGURE 7 | *In situ* location of the main classes of the chemical compounds detected in cross sections of nectariferous disks and in nectar of *Jacaranda oxyphylla* flowers. **(A–C)** Positive reaction to Lugol's iodine showing progressive depletion of starch grains during anthesis (0, 24, 48 h flowers, respectively); **(D)** phenolic inclusions (in green) with toluidine blue staining (48 h flowers); **(E)** positive reaction to Sudan IV for lipids (arrow) (48 h flowers); **(F)** positive reaction to NADI's reagent for essences (arrow) (48 h flowers); **(G)** positive reaction for lipids in raw floral nectar with Sudan Black B (48 h flowers).

Nectarless flowers

At 0 h of anthesis, the epidermal and parenchyma cells exhibited variable sizes and shapes, and variable cytoplasmic densities (**Figure 11A**). Epidermal cells were thick-walled and covered with a continuous thin cuticle. In the cytoplasm, SER profiles and mitochondria were the most evident organelles (**Figure 11B**). The first subepidermal parenchyma layer consisted of thin-walled expanded cells, with cytoplasm reduced to a thin parietal layer (**Figure 11B**). Amyloplasts were uncommon in these cells, while lipophilic inclusions were abundant and occurred adhered to the inner surface of the tonoplast in epidermal and subepidermal cells (**Figure 11B**). The subsequent two to three parenchyma layers were composed by axially elongated cells that differed from those of the first subepidermal layer regarding cytoplasmic density and vacuole system development. In this disk region, clusters of two to three cells with smaller sizes and characterized by thinner walls, prominent nucleus, abundant cytoplasm, and poorly developed vacuoles were common (**Figure 11C**). The occurrence of small amyloplasts with prominent starch grains and large mitochondria was common in newly derived cells (**Figure 11F**).

At 24 h of anthesis, the most remarkable difference in relation to the previous stage was the occurrence of protuberances on the epidermal cells' outer tangential walls (**Figures 11D,E**). Moreover, large cells that at the previous stage were located in the subepidermal position (**Figures 11A,B**), now appeared interspersed with epidermal cells. This aspect was also observed in histological sections (**Figure 6F**). In addition, osmiophilic materials were more abundant at this stage and could be observed on the cuticle surface and mainly in periplasmic space (**Figure 11E**).

Subepidermal parenchyma cells, at both 0 and 24 h of anthesis, had similar ultrastructural organization, characterized by scarce amyloplasts with few or lacking starch grains. The coexistence of plastids with distinct morphologies and/or inclusions was common in the same or neighboring cells. The most common type of plastids was rounded, devoid of thylakoids, filled with electron-dense phenolic content and containing small starch grains (**Figures 11C,G,I**). Oval-shaped plastids with reduced or lacking starch grains and large oil inclusions from which oil drops flow toward the cytoplasm and vacuoles were also observed (**Figure 11H**). Oil drops occurred near the plastids (**Figure 11I**). Dimorphic plastids having conspicuous pressed starch grains on one of its poles and, vesicle/tubular membranes on the opposite pole devoid of starch grains (**Figure 11J**), occurred in the interface between subepidermal and deeper parenchyma layers. Elongated, undifferentiated plastids were common in these cells

TABLE 1 | Histochemical tests on the secretory disk in nectariferous and nectarless flowers of *Jacaranda oxyphylla* (Bignoniaceae).

Staining procedure	Target compounds	Positive reaction site	
		Nectariferous flowers	Nectarless flowers
Sudan IV	Total lipids	Cuticle, subcuticular space, cell wall, cytoplasm, and vacuole (EP, NP, SN)	Cuticle, subcuticular space, cell wall, cytoplasm, and vacuole (EP, NP, SN)
Ferric chloride	Phenolic compounds	Amyloplasts and vacuole (EP, NP, SN)	Amyloplasts and vacuole (EP, NP, SN)
Lugol's iodine	Starch	Amyloplasts and vacuole (EP, NP, SN)	Amyloplasts and vacuole (EP, NP, SN)
NADI's reagent	Essences	On the cuticle, subcuticular space, cytoplasm, vacuole (EP, NP, SN)	On the cuticle, subcuticular space, cytoplasm, vacuole (EP, NP, SN)

EP, epidermis; NP, nectary parenchyma; SN, sub-nectary parenchyma.

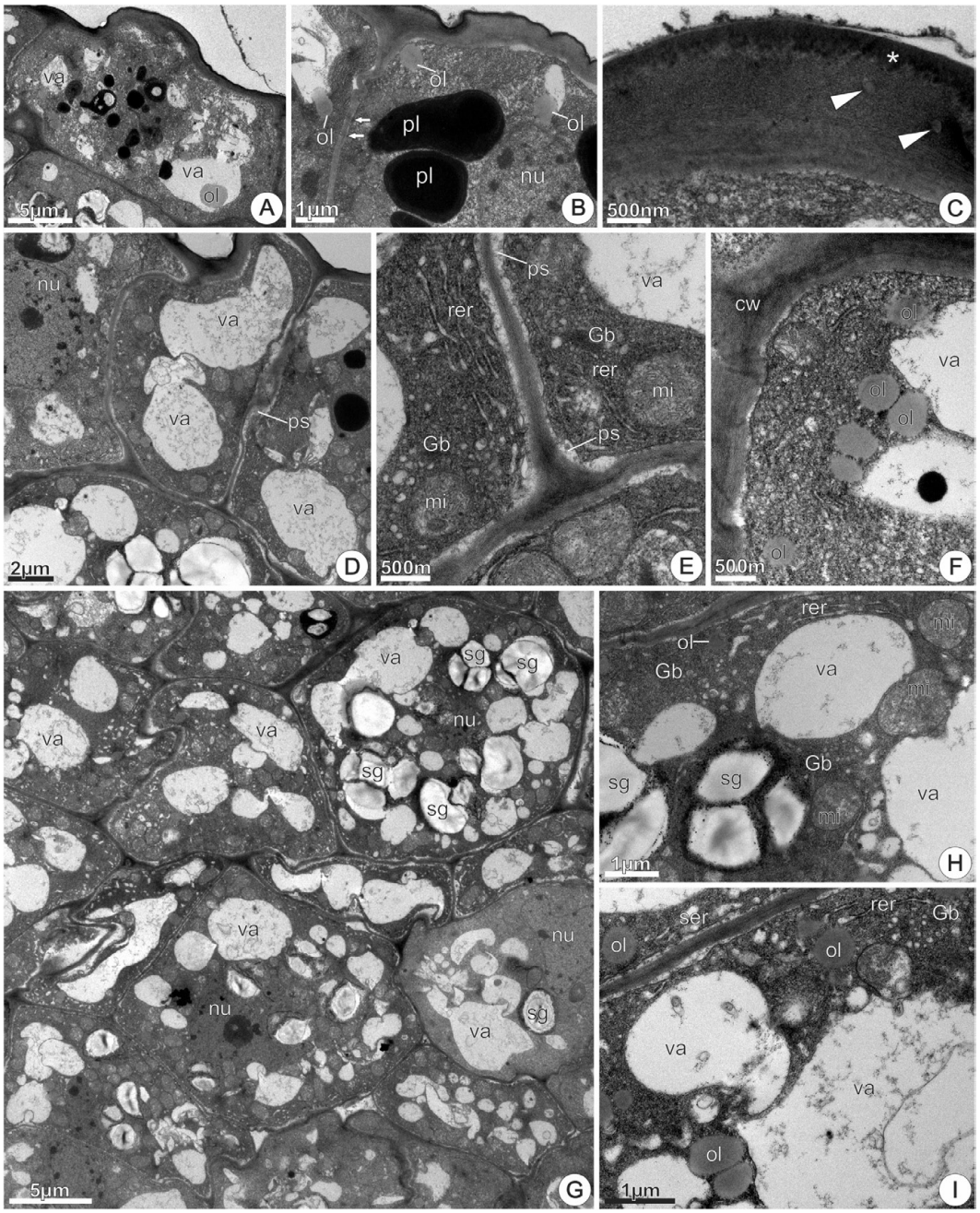

FIGURE 8 | TEM micrographs of the disk from *Jacaranda oxyphylla* nectariferous flowers, at 0 h of anthesis. **(A–F)** Epidermal cells. **(A)** Rectangular cells with cytoplasm and vacuoles with heterogeneous inclusion; **(B)** plasmodesmata (arrows), conspicuous nucleus, abundant cytoplasm with dense plastids and oil drops; **(C)** detail of the outer tangential cell wall highlighting oil inclusions (arrow heads) embedded in the wall matrix and osmiophilic deposits (asterisk) in the cuticular layer; **(D)** conspicuous nucleus, developed vacuoles, sinuous plasmalemma, and small periplasmic spaces; **(E)** detail of **(D)** showing abundance of organelles in the peripheral cytoplasm and flocculent materials inside vacuole and periplasmic space**; (F)** polyribosomes through the cytosol and oil bodies adjacent to tonoplast. **(G–I)** Nectary parenchyma. **(G)** General aspect showing cells side by side with different ultrastructure; **(H)** detail of **(G)** showing amyloplasts with residual starch grains and RER profiles assembled around degenerating amyloplasts and vacuoles; **(I)** vacuoles with flocculent materials and membrane debris, hyperactive Golgi body, enlarged SER elements and oil drops. Gb, Golgi body; mi, mitochondria; nu, nucleus; ol, oil; pl, plastid; ps, periplasmic space; rer, rough endoplasmic reticulum; ser, smooth endoplasmic reticulum; sg, starch grains; va, vacuole.

(**Figure 11K**). In the deeper parenchyma layers (**Figure 11L**), all the plastids contained conspicuous starch grains and were larger than those in subparenchyma layers.

At 48 h of anthesis, the features of the subepidermal layers remained similar to the observed at the previous stage (not shown here). The most remarkable difference occurred in the deeper

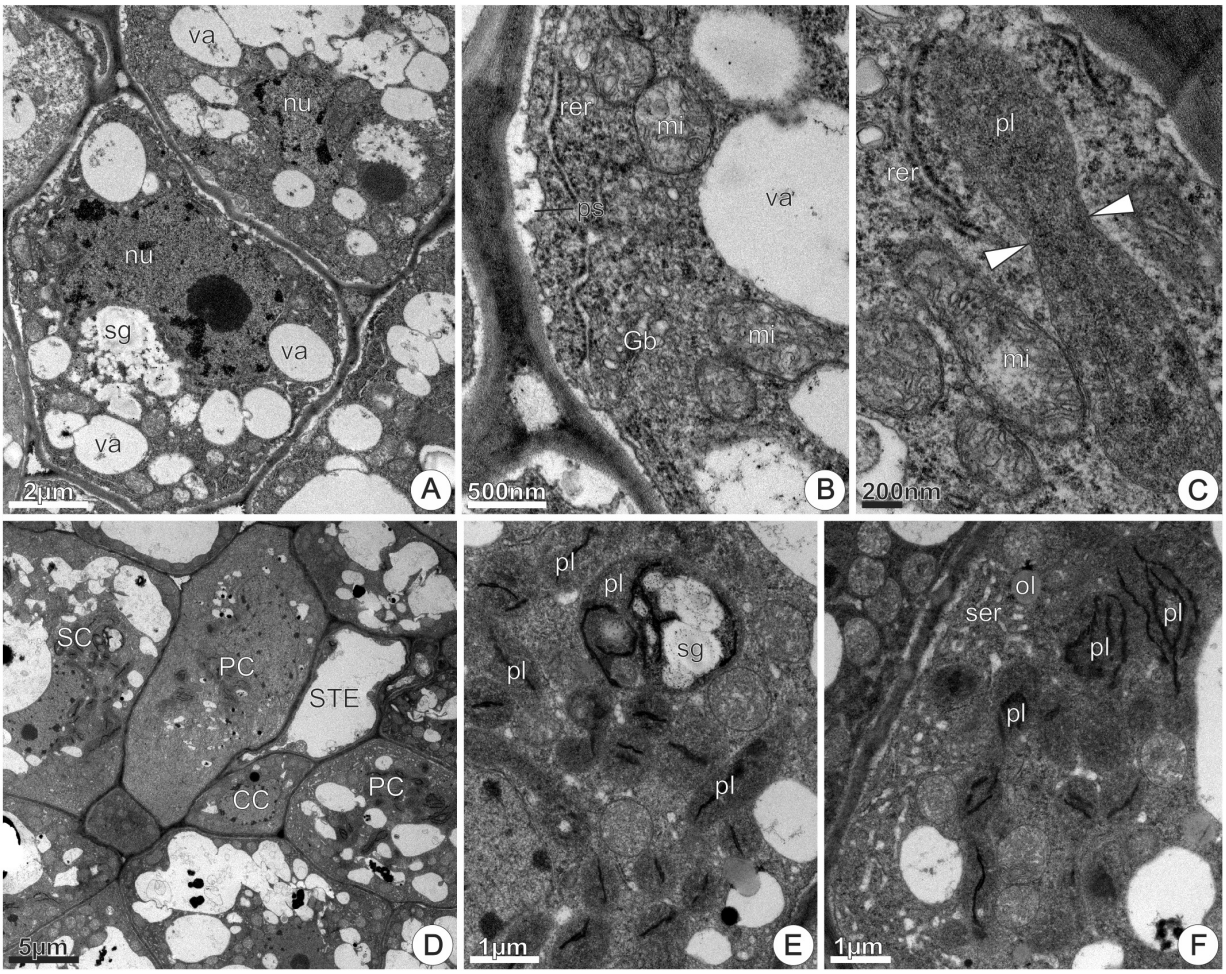

FIGURE 9 | TEM micrographs of the disk from *Jacaranda oxyphylla* nectariferous flowers, at 24 h of anthesis. **(A–C)** Nectary parenchyma. **(A)** General aspect showing vacuolated cells with prominent nucleus and evident nucleolus, degenerating amyloplast and merged vacuoles; **(B)** detail of **(A)** showing cytoplasm rich in polyribosomes, mitochondria, extensive RER and Golgi bodies located in the peripheral cytoplasm, and developed periplasmic spaces; **(C)** elongated plastid with clear median constriction (arrowheads) surrounding with RER elements and mitochondria. **(D–F)** Subnectary parenchyma. **(D)** General view of the subnectary parenchyma and phloem cells; **(E)** detail of (D) showing chloro-amyloplasts and undifferentiated plastids in parenchyma cell; **(F)** plastids with thylakoid-like membranes and dilated SER elements assembled in the peripheral cytoplasm of phloem parenchyma cell. CC, companion cell; Gb, Golgi body; mi, mitochondria; nu, nucleus; ol, oil; PC, phloem parenchyma cell; pl, plastid; ps, periplasmic space; rer, rough endoplasmic reticulum; ser, smooth endoplasmic reticulum; sg, starch grains; SC, subnectary parenchyma cell; STE, sieve tube element; va, vacuole.

parenchyma layers, which showed an increase in the amount and volume of starch grains in plastid profiles (**Figures 12A–C**), besides accumulations of black granulations in the intercellular space (**Figure 12A**). In addition, parenchyma cells exhibited dense cytoplasm and large oil bodies (**Figure 12B**). The progressive increase in accumulations of phenolic compounds in the amyloplasts was also noticeable (**Figures 12B–D**). Signs of amyloplasts degeneration and hydrolysis of the starch grains were commonly observed, and plastids debris, including phenolic compounds, were seem inside vacuoles (**Figure 12D**). The vacuoles merged together forming larger vacuoles on cell periphery (**Figure 12E**), pushing the remaining amyloplasts toward the nucleus, which occupies a central position. Also, in these cells, clusters of globular mitochondria around the vacuoles or degenerating amyloplasts were common (**Figure 12F**). Chloro-amyloplasts with undeveloped thylakoids were commonly found at this stage (**Figure 12G**). These plastids contained small globular starch grains, abundant stroma and were devoid of phenolic compounds.

Volatile Compounds Common to Floral Disk and Floral Scent

The chemical analyses of the floral disks revealed that the disks of nectariferous flowers had no volatile compounds in common with *in situ* floral scent. In contrast, the disks of nectarless flowers presented four volatile compounds that were also present in *in situ* floral scent. Three of them were the aliphatic compounds tridecane, tetradecane, and hexadecane, and one was the aromatic compound phenylacetaldehyde (**Table 2**). We registered a variation in the presence of

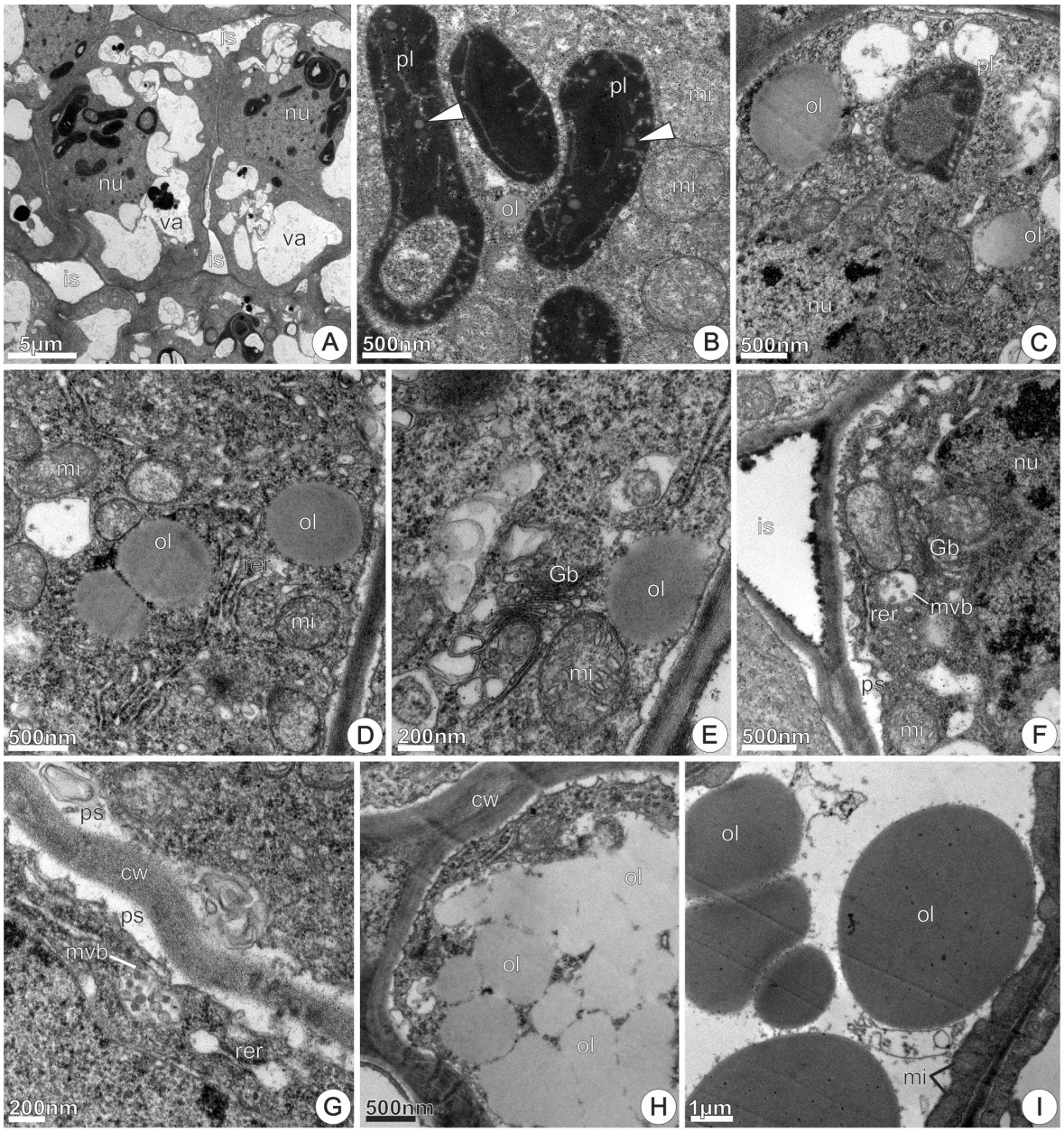

FIGURE 10 | TEM micrographs of the disk from *Jacaranda oxyphylla* nectariferous flowers, at 48 h of anthesis. **(A)** General view of the nectary parenchyma showing larger intercellular spaces and parenchyma cells with prominent nucleus and dense cytoplasm; **(B)** polymorphic plastids with electron-dense stroma, vesicular/tubular inner membranes and lipid globules; **(C)** large oil droplets, RER elements and mitochondria scattered through the cytosol; **(D)** part of a nectary parenchyma cell highlighting the conspicuous nucleus, modified plastid and oil droplets; **(E)** oil drop near the plasmalemma, numerous polyribosomes and hyperactive Golgi body; **(F)** mitochondria and RER elements positioned in the peripheral cytoplasm, sinuous plasmalemma and dense granulations inside intercellular space; **(G)** part of two nectary parenchyma cells highlighting multivesicular body in close juxtaposition with the plasmalemma that is sinuous, and developed periplasmic space containing dense granulations and multilamellar membranes; **(H)** assemblage of oil drops in the cytoplasm; **(I)** large oil bodies in the vacuole, and clustered mitochondria in the reduced parietal cytoplasm. cw, cell wall; Gb, Golgi body; is, intercellular space; mi, mitochondria; mvb, multivesicular body; nu, nucleus; ol, oil; pl, plastid; ps, periplasmic space; rer, rough endoplasmic reticulum; sg, starch grains; va, vacuole.

these compounds in the headspace samples, with one sample showing all the four compounds, two samples showing only phenylacetaldehyde and tridecane, one sample showing only tetradecane and hexadecane and one showing none of them.

DISCUSSION

In this study, we characterized the spatial and temporal variation in nectar production and compared disk histology, chemistry and cellular features in nectariferous and nectarless flowers,

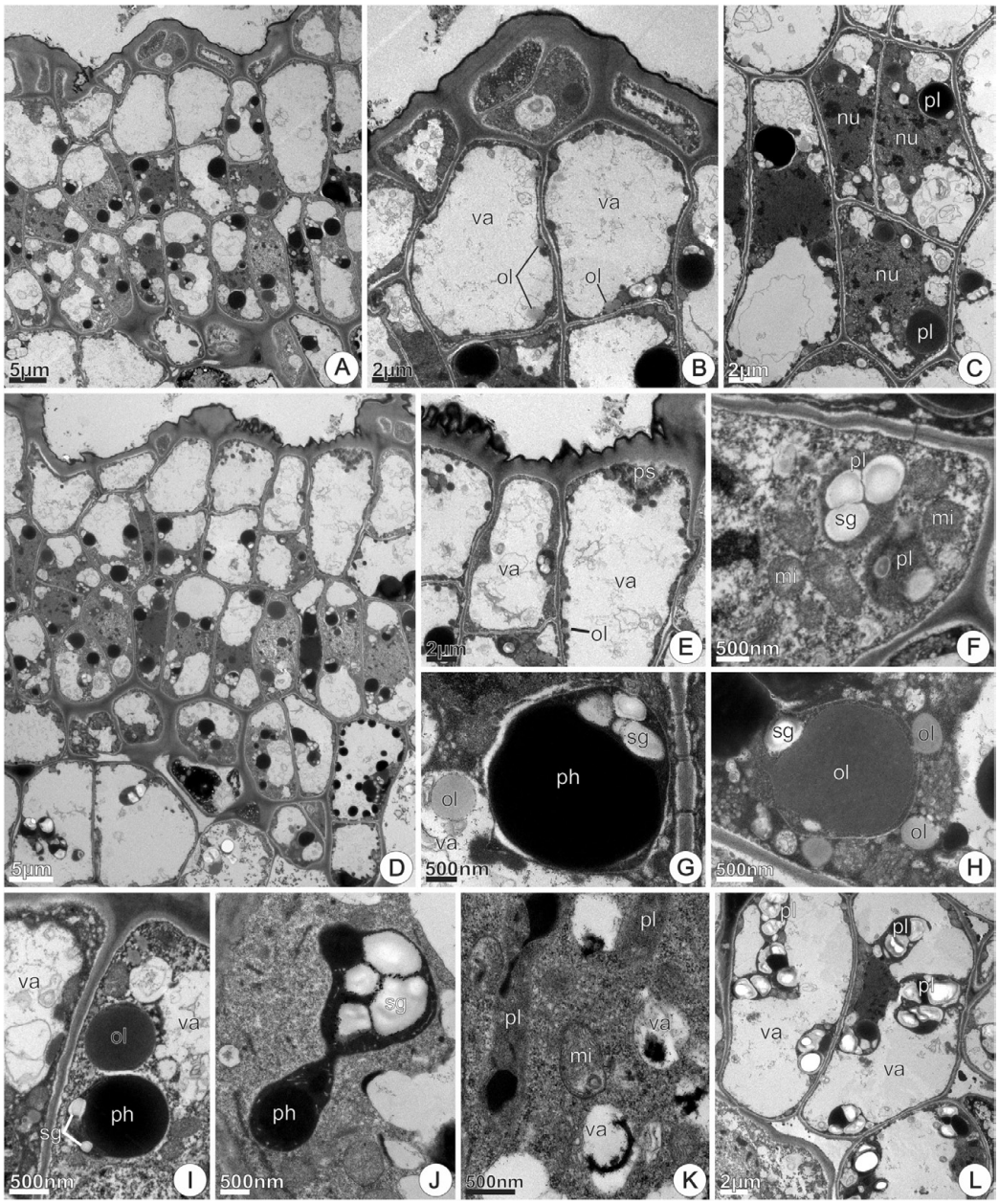

FIGURE 11 | TEM micrographs of the disk from *Jacaranda oxyphylla* nectarless flowers. **(A–C,F)** At 0 h of anthesis. **(A)** General view showing irregular epidermis and juxtaposed subepidermal cells; **(B)** detail of **(A)** highlighting epidermal cells with dense cytoplasm, highly vacuolated subepidermal cells and osmiophilic inclusions facing the inner surface of the tonoplast; **(C)** cluster of three newly derivate subepidermal cells showing voluminous nucleus and rounded plastids with dense inclusions. **(D,E,G–L)** At 24 h of anthesis. **(D)** General view showing vacuolated parenchyma cells interspersed with detached epidermal cells; **(E)** detail of **(D)** showing osmiophilic droplets inside vacuole and periplasmic space besides secretions on the cuticle; **(F)** amyloplast with globular starch grains and large mitochondria; **(G)** rounded plastid containing small starch grains and phenolic content, and lipid drop in the vacuole; **(H)** oval plastid filled with lipid content from which oil drops flow toward the cytoplasm; **(I)** large oil drop near plastid with reduced starch grains; **(J)** dimorphic plastid with starch grains and phenolic content in the opposite poles, showing a constriction in their median region; **(K)** undifferentiated plastids and vacuoles containing dense inclusions; **(L)** general view of subnectary parenchyma showing vacuolated cells with prominent amyloplasts. mi, mitochondria; nu, nucleus; ol, oil; pl, plastid; ph, phenolic content; sg, starch grains; va, vacuole.

which corresponded to 53 and 47% of *J. oxyphylla* flowers, respectively. We found a broad spatial variation in nectar volume in nectariferous flowers, including intra- and inter-plant differences. Additionally, we also found temporal variation in nectar production, with 31% of the nectariferous flowers presenting the higher nectar volume at the moment of flower opening ('early' flowers) and 22% presenting it only in the second day of anthesis ('late' flowers). Both nectariferous flower

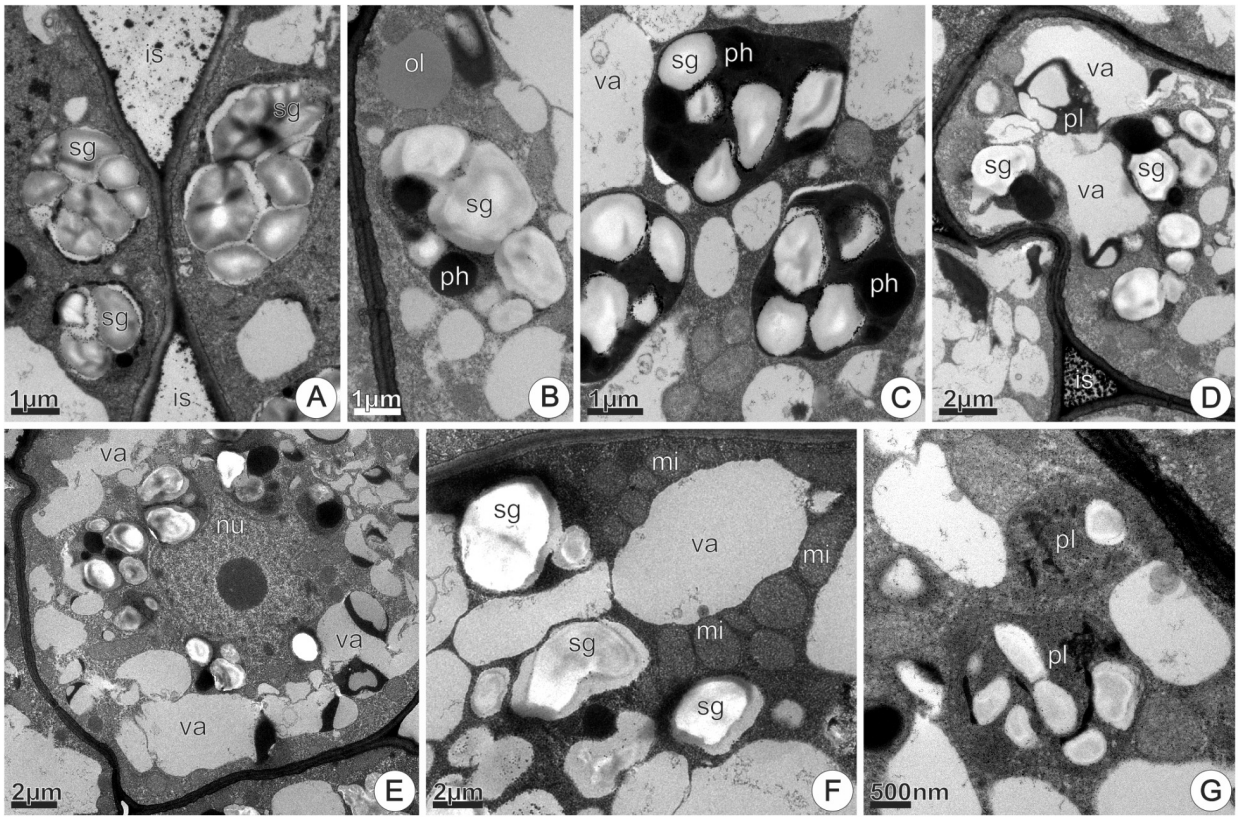

FIGURE 12 | TEM micrographs of the disk from *Jacaranda oxyphylla* nectarless flowers, at 48 h of anthesis, highlighting the plastids change in the deeper parenchyma layers. **(A)** Bigger amyloplasts in the periphery of the cell and black granulations adhered in the cell walls bounding the intercellular space; **(B)** amyloplast with voluminous starch grains and phenolic inclusions. Note oil drop in the cytosol; **(C)** denser amyloplasts with signs of starch hydrolysis; **(D)** degenerating amyloplast engulfed in the vacuole; **(E)** merged vacuoles from amyloplasts degeneration in the periphery of the cell, and amyloplasts clustered around the nucleus; **(F)** mitochondria assembled around vacuoles; **(G)** Chloro-amyloplasts with undeveloped thylakoids. is, intercellular space; mi, mitochondria; nu, nucleus; ol, oil; pl, plastid; ph, phenolic content; sg, starch grains; va, vacuole.

types, 'early' and 'late,' exhibited cellular apparatus typical of nectar secretion, showing a continuous decrease of starch grains' size and number during the first 48 h of anthesis. Although nectariferous and nectarless flowers showed similar histological organization, at cellular level, nectarless flowers exhibited osmophoric features. In fact, the disks of nectarless flowers showed volatile compounds that were also present in floral scent of *J. oxyphylla*, suggesting its participation in floral chemical signaling. In addition, disks in nectariferous flowers seem to play a dual function, secreting predominantly nectar in the first 48 h of anthesis and only lipophilic substances from this time on.

TABLE 2 | Absolute amounts (mean ± SE) of scent compounds found in *Jacaranda oxyphylla* (Bignoniaceae) floral disks (ng.disk^{-1}) and *in situ* floral headspace (ng.flower^{-1}; 30 min^{-1}).

		Floral disks		
Compounds	**RI**	**Nectarless**	**Nectariferous**	**Flowers**
Aliphatic compounds				
Tridecane	1300	23.54	–	0.36 ± 0.04
Tetradecane	1400	29.25	–	0.63 ± 0.05
Hexadecane	1600	64.14	–	0.69 ± 0.49
Aromatic compounds				
Phenylacetaldehyde	1045	63.73	–	0.65 ± 0.23

RI, Kovats retention index.

Nectar Production Variability in Space and Time

Nectar volume variation at plant or population level, in general, could be caused by the nectarivores, which would empty flowers as they forage, or by plant species characteristics (Heinrich, 1975; Feinsinger, 1978; Brink and deWet, 1980; Zimmerman, 1981), such as the variable presence of nectariferous and nectarless flowers among plants (Gervasi and Schiestl, 2017). Both cases create heterogeneity in the resource availability to pollinators in natural populations, in a way that pollinators could or could not react to it (Real, 1981). The nectar variability reported in this study could be responsible for the scarce pollinator visits and, consequently, for the low reproductive success described for *J. oxyphylla* by Guimarães et al. (2008). On the other

hand, nectarless flowers could favor cross-pollination, as they may coerce pollinators to visit other plants after encountering some empty flowers (Thakar et al., 2003). This scenario could be especially relevant in a self-incompatible species, such as *J. oxyphylla* (Guimarães et al., 2008) since it may reduce geitonogamy and favor allogamy (Johnson, 2000).

The occurrence of nectarless flowers has been associated with high-density plant populations (Thakar et al., 2003; Anand et al., 2007; Zhao et al., 2016), which is the case of *J. oxyphylla* that showed clumped distribution in the study natural population. The presence of nectarless flowers in natural populations may represent advantages from the plants' perspective (Thakar et al., 2003 and references therein), especially, in plant species with concealed nectar, as *J. oxyphylla*, which are most likely to present highly variable nectar volume (Bell, 1986). As pollinators have no visual cue to predict the presence or absence of nectar in a flower before trying it (Smithson and Gigord, 2001), it is expected that they would not exert selective pressures toward nectar volume stabilization. Therefore, these species might present a higher proportion of flowers without nectar or with very low volumes, supporting the idea that those plant species cheat on pollinators (Bell, 1986; Gilbert et al., 1991). In fact, even though some Bignoniaceae species have complex floral nectaries (Lopes et al., 2002; Machado et al., 2017b) and produce large amounts of nectar (Cruden et al., 1983), others have no nectaries (Alcantara and Lohmann, 2010) or nectar, being pollinated by deceit (Umaña et al., 2011). However, such an expressive intraspecific variation in nectar production has never before been reported for any Bignoniaceae species.

A variety of plant species, which are known to produce nectarless flowers, are believed to be pollinated through Batesian mimicry (Firmage and Cole, 1988; Johnson, 1994, 2000; Gigord et al., 2002; Juillet et al., 2007). Additionally, nectarless species could benefit from cheating on naïve pollinators (Gigord et al., 2002). The mimicry idea could also apply to plant species that present individuals having only nectariferous flowers and only nectarless flowers in the same population, or even mixed proportions of nectariferous and nectarless flowers in the same individuals, as does *J. oxyphylla*. Plants that present only nectarless flowers could have lower reproductive success when compared to plants with only nectariferous flowers and with mixed flower types, because pollinators can learn to avoid them (Smithson and MacNair, 1997; Ferdy et al., 1998; Gumbert and Kunze, 2001; Vásquez and Barradas, 2018). Besides the heterogeneity of nectar production within and among plants in *J. oxyphylla*, our results also showed high heterogeneity among nectariferous flowers as well, which is showcased by the fact that we found two types of nectar production rhythms ('early' and 'late' flowers). The causes of this variation in nectar production rhythm in *J. oxyphylla* flowers are yet unknown. Most bee-pollinated Bignoniaceae species start nectar production before anthesis (Galetto, 1995; Lopes et al., 2002; Maués et al., 2008; Guimarães et al., 2016; Quinalha et al., 2017; Souza et al., 2017), so that pollinators have high probability of finding nectar in freshly opened flowers. However, in *J. oxyphylla*, when searching for nectar in recently opened flowers, pollinators have a 78% chance of finding empty flowers, considering that 47% of flowers are nectarless and 31% start nectar release just in the second day of anthesis ('late' flowers).

Nectar reabsorption is a common phenomenon in angiosperms (Torres and Galetto, 1998; Stpiczyńska, 2003; Nepi and Stpiczyńska, 2008; Antoń et al., 2017), which has been considered as resource-recovery strategy (Nepi and Stpiczyńska, 2008). One might have thought that the occurrence of nectarless flowers in *J. oxyphylla* would be a sign of nectar reabsorption; however, our results showed that there was no decrease in the accumulated nectar volume throughout anthesis and no difference between the accumulated volume and the sum of daily nectar production. These findings together with the wide window of monitoring flowers (144 h) showed that, undoubtedly, nectarless flowers have no nectar from start to finish of anthesis, and that nectar reabsorption is not the cause of it.

Histological, Histochemical, and Ultrastructural Features of the Floral Disk

According to our results, there are no significant histological and histochemical differences between the disks of nectariferous and nectarless flowers of *J. oxyphylla*. However, ultrastructural observations allowed us to identify differences concerning their fine structure and functioning. Our histochemical and utrastructural analysis suggest that the disk of nectariferous flowers has a dual function, wherein until 48 h of anthesis it produces predominantly nectar and in subsequent stages of anthesis, lipophilic secretion is predominant. Moreover, the ultrastructural analysis provided clear evidence to conclude that the disk of nectarless flowers has secretory activity associated with lipophilic secretion during the whole anthesis.

The disk in nectariferous flowers of *J. oxyphylla* showed typical characteristics of nectary tissues, such as small thin-walled cells, large nuclei, small vacuoles and dense cytoplasm (Fahn, 1979; Nepi, 2007; Guimarães et al., 2016). The ultrastructural features observed in nectariferous flowers at the beginning of anthesis (at 0 and 24 h) are similar to those reported for nectaries of other angiosperms and are indicative of high metabolic activity (Fahn, 1979; Nepi, 2007; Guimarães et al., 2016; Machado et al., 2017b). The subcellular changes observed in these flowers at this moment of anthesis, mainly the alterations in amyloplasts, are involved in the conversion of starch to nectar, as reported for other angiosperm species (e.g., Fahn and Shimony, 2001; Nepi, 2007; Paiva and Machado, 2007; Guimarães et al., 2016; Machado et al., 2017b). The juxtaposition of large mitochondria with amyloplasts may be related to energy requirements during starch hydrolysis (Fahn, 1979). The occurrence of polyribosomes, well-developed Golgi bodies and RER elements can also be related to the synthesis of enzymes involved in starch grains' hydrolysis and degradation processes, which were observed during nectar secretion. Moreover, RER elements may also be involved in translocation and/or temporary concentration of sugars (Durkee, 1983; Figueiredo and Pais, 1992; Paiva and Machado, 2007). The occurrence of vesicles close or fused to plasmalemma, and the formation of ample periplasmic spaces suggest that the elimination of secretion products from the protoplast

occurs by exocytosis (Fahn, 1979; Nepi, 2007; Machado et al., 2017b).

Our data indicate that the production of lipophilic substances increased throughout the anthesis in nectariferous flowers. This data is compatible with the predominance of elaioplasts in the nectary parenchyma cells at 48 h of anthesis, when most of amyloplasts are degenerated and nectar secretion has already stopped. Plastid change occurred simultaneously with the increase in the amount of oil drops inside vacuoles or dispersed in the cytosol, and SER proliferation, which are ultrastructural evidences of lipophilic secretion (Gleizes et al., 1980; Figueiredo and Pais, 1992; Turner et al., 1999; Machado et al., 2005; Stpiczyńska et al., 2005; Stpiczyńska and Davies, 2016; Possobom and Machado, 2018). Although these features are unusual in nectary tissues, the ability of nectary cells to produce both nectar and lipids (Baker and Baker, 1975; Durkee et al., 1984; Subramanian et al., 1990; Possobom et al., 2010; Tölke et al., 2015; Guimarães et al., 2016; Stpiczyńska and Davies, 2016; Machado et al., 2017b) and transition from a true nectary to a lipophilic secretory gland (Durkee, 1982; Durkee et al., 1984) has been reported in some angiosperm species.

Our histological and ultrastructural observations revealed that the disk from nectarless flowers had subcellular evidences of secretory activity associated to the production of volatile substances. The histological characteristics exhibited by the disk, as an irregular surface that enhances the area of secretion release, highly vacuolated epidermal cells, compact arrangement of subepidermal tissue with several layers of depth and vascularization with vein endings consisting of phloem only, are common to osmophores, according to Vogel (1990).

Some authors, studying Orchidaceae species (de Melo et al., 2010; Kowalkowska et al., 2012, 2015; Wiśniewska et al., 2018), found that floral nectaries and osmophores are somewhat similar in structure and ultrastructure features, except that in the latter there is predominance of SER and low frequency of Golgi bodies. Therefore, abundance of globular mitochondria, SER with peripheral distribution and oil droplets in the cytosol and in amyloplasts, together with the scarce Golgi bodies here observed may be associated with fragrance production (Curry et al., 1991; Stpiczyńska and Davies, 2016). The occurrence of polymorphic plastids containing many lipophilic droplets and numerous large oil inclusions in the cytoplasm of parenchyma cells both in nectariferous flowers at 48 h of anthesis and in nectarless flowers is a strong evidence of the involvement of the floral disk in scent production, as similar plastids also occur in osmophores (Pridgeon and Stern, 1983, 1985; Curry et al., 1991; de Melo et al., 2010; Antoń et al., 2012).

Although amyloplasts are common components of both nectaries and osmophores (Nepi, 2007), the absence of starch has been recorded in osmophores in some orchid species (Wiśniewska et al., 2018). Plastids lacking or having reduced starch grains in the epidermis and in the first subepidermal layers at recently opened nectarless flowers, as here observed, might be caused by their hydrolysis before anthesis (at an earlier stage than we sampled), as starch grains are utilized as energy source for scent production (Stern et al., 1987; Vogel, 1990; Nepi, 2007; Pacini and Nepi, 2007; de Melo et al., 2010).

Small droplets of lipophilic material in the disk epidermal cells that stained with Sudan IV and Nadi reagent, and also observed in TEM analysis, were reported in osmophore epidermal cells of several Orchidaceae species (Davies and Stpiczyńska, 2014; Stpiczyńska and Davies, 2016) and indicate the possible role of the epidermal cells in scent production. In addition, a layer of osmiophilic material lined the tonoplast inner surface, as occurs in the epidermal and parenchyma vacuole of *J. oxyphylla* nectarless flowers is also evidence of scent production.

The emission of volatile substances is of short duration and is associated with the fast utilization of large amounts of starch grains (Stern et al., 1987; Vogel, 1990; Pacini and Nepi, 2007; de Melo et al., 2010; Wiśniewska et al., 2018). In fact, essences were histochemically detected since the beginning of anthesis in nectarless flowers. In fact, prominent periplasmic space containing lipid droplets in regions underlying the outer tangential walls indicate secretory activity of epidermal cells associated with the accumulation and release of the volatile compounds. Likewise, the occurrence of large mitochondria and abundant SER located in the cortical cytoplasm, besides plastids featured by a reduced electron density and lipid inclusions, together with numerous lipid droplets in the cytosol and vacuoles of the subepidermal layers, are subcellular features commonly associated with synthesis of volatile compounds (Cheniclet and Carde, 1985; Figueiredo and Pais, 1994; Ascensão et al., 1997; Turner et al., 1999; Possobom et al., 2015; Stpiczyńska and Davies, 2016).

A noteworthy feature of the disks from nectarless flowers was the coexistence of plastids with different inclusions, only lipids or combinations of carbohydrate and phenolic substances. The increase in the amount of different materials (lipid–carbohydrate–phenolic) within the plastids throughout anthesis, as here observed, has been demonstrated in Orchidaceae osmophores (Wiśniewska et al., 2018 and references therein). The involvement of plastids in the synthesis of scent components, mainly terpenoids and phenolic compounds, has been broadly discussed (Pridgeon and Stern, 1985; Stern et al., 1987; Pais and Figueiredo, 1994; Kowalkowska et al., 2012; Stpiczyńska and Davies, 2016; Wiśniewska et al., 2018). Besides protection against herbivores, pathogens, and UV radiation (Brillouet et al., 2013), phenolic compounds are known to occur in floral scent (Jürgens and Dötterl, 2004; Jürgens et al., 2006; Steiner et al., 2011; Wiśniewska et al., 2018).

Our ultrastructural observations suggest that volatile compounds produced in plastids, cross the plastid envelope to the profiles of SER or migrate independently in the cytosol, and finally reach the plasmalemma and leave the protoplast by eccrine mechanism (Fahn, 1979). Volatile compounds participate in the attraction of mutualists or in the deterrence of antagonists (Harborne, 1997; Raguso, 2004; Guimarães et al., 2008). The most remarkable feature of the ground parenchyma cells in the disk of nectarless flowers after 72 h of anthesis was the presence of amyloplasts with prominent starch grains and dense phenolic inclusions, together with amyloplasts with signs of starch hydrolysis, and large vacuoles containing membrane debris, flocculent materials and phenolic compounds that were engulfed in the vacuoles, as discussed earlier. Therefore, we suggest that

the occurrence of continuous storage and successive degradation of starch grains allowed continued production of volatiles throughout nectarless flowers' disks lifespan.

The floral disk was supplied only by phloem, and it is probable that pre-nectar from the sieve tubes move away from an apoplastic route via intercellular spaces and from cell walls (e.g., Wist and Davis, 2006). Afterward, stored starch is progressively hydrolyzed and polysaccharides are transported from the amyloplasts to the vacuoles by vesicles. Accumulation of dense material in the periplasmic spaces, cell walls and intercellular spaces of nectary parenchyma tissue suggest that the apoplast system is involved in nectar transportation (Nepi, 2007) in *J. oxyphylla*.

The occurrence of modified stomata with associated secretions across the entire epidermis of the floral disk indicates the site of secretion release to the disk surface (Gaffal et al., 1998; Wist and Davis, 2006). In addition, secretion seems to also be released through microchannels in the cuticular layer, as cuticle channels may increase porosity and facilitate the passage of macromolecules (Rocha and Machado, 2009; de Melo et al., 2010; Stpiczyńska et al., 2010; Machado et al., 2017b).

In addition, a symplastic pathway of pre-nectar could also occur in the floral disk via plasmodesmata, which connects parenchyma and epidermal cells (Vassilyev, 2010). Moreover, the presence of well-developed Golgi bodies, numerous profiles of RER secretory vesicles originated from RER or Golgi bodies close to the plasmalemma that fuses with it, plasmalemma sinuous in outline, and developed periplasmic space, indicate vesicle-mediated process of secretion, providing evidence of a granulocrine mechanism of nectar release (Fahn, 1979). For lipophilic substances, as they are commonly present as droplets close to the plasma membrane or inside the periplasmic spaces, there is evidence of eccrine mechanism (Fahn, 1979), where the molecules cross the plasma membrane by active transport (Vassilyev, 2010). Both mechanisms of secretion release from the protoplast seem to occur simultaneously in the disk of nectariferous flowers, while in nectarless flowers, the eccrine mechanism is predominant.

Evidences of cell divisions in the nectary parenchyma, with the newly derivate cells integrating into the secretory tissue, were a common cytological feature to nectariferous and nectarless flowers. This process, quite similarly to that which occurs in meristematic tissues, has been reported in nectaries (Nepi, 2007) and secretory canals and cavities (Machado et al., 2017a) revealing the regenerative potential of secretory cells.

Although the occurrence of chloro-amyloplasts with undeveloped inner membranes in the subnectary parenchyma is an ultrastructural indication of the production of carbohydrates (Nepi, 2007), we have strong evidences that the carbohydrate supply comes largely from the hydrolysis of starch grains stored in amyloplasts. Diversity of plastids types, as here observed, including plastids with starch grains, undifferentiated plastid with osmiophilic bodies, chloro-amyloplasts, chloroplasts with poorly developed thylakoids or plastids with thylakoid-like membranes are common in floral nectaries of different taxa, mainly orchid species (Nepi, 2007). In a general way, undifferentiated plastids occur in the very early stages of nectary development, undergo some divisions before beginning to differentiate (Pacini et al., 1992; Nepi et al., 1996) and close to flower anthesis, chloro-amyloplasts are generally present in nectary parenchyma when secretion begins (Nepi, 2007). Contrary to previously investigated species, in the present study, undifferentiated plastids (or proplastids) and evidences of plastids division, were detected in nectary parenchyma cells at 48 h of anthesis, when nectar release stopped and amyloplasts were degenerated. A similar pattern of plastid differentiation was verified in nectarless flowers throughout anthesis. Based on this, we speculate that in *J. oxyphylla* there is no conversion of amyloplasts in elaioplasts, but differentiation of proplastids throughout the flowers lifespan.

Chemical Composition of Floral Disks, of Secretion and Ecological Implications

The chemical analysis revealed that the nectarless disks do in fact produce volatile compounds. Generally, the disks might produce compounds that represent the floral scent as a whole or they might produce volatiles that add to an overall more complex scent (Dötterl and Jürgens, 2005; Dötterl and Vereecken, 2010). In present study, the compounds detected in the disk samples were also detected in floral headspace, and it remains to be tested how the disk contributes to the floral headspace and whether other flower organs also release these components. Nevertheless, detected compounds might have an impact on pollinator attraction in spite of nectarlessness. Indeed, all four compounds have been reported to be released by other bee-pollinated plants (e.g., Knudsen et al., 2006; Steiner et al., 2011) and phenylacetaldehyde is even a known attractant for bees of different families (Dötterl and Vereecken, 2010). This suggests that especially phenylacetaldehyde, but potentially also the other compounds, are involved in attracting the bee pollinators of *J. oxyphylla*. Bees are known to use floral volatiles to discriminate among resourceful and resourceless flowers of a given species (Dötterl and Vereecken, 2010). Thus, especially naïve bees might be cheated by the scent and visit nectarless flowers.

Even though plants with nectarless flowers are said to take advantage of higher fitness as they do not spend energy on nectar production (Thakar et al., 2003), there may be a counterbalance in this scenario, since pollinators may avoid visiting these flowers based on previous learning of nectar absence (Smithson and MacNair, 1997; Ferdy et al., 1998; Gumbert and Kunze, 2001; Vásquez and Barradas, 2018). In our focus species, approximately half of the flowers did not produce nectar, but produced volatile substances. These compounds could have a role in the maintenance of this plant–pollinator interaction, counterweighing the reduced attractiveness of resourceless flowers. So, *J. oxyphylla* might be taking a different path than Bignoniaceae species in which the complete loss of the ability to produce nectar was associated to disk loss (Umaña et al., 2011).

Additionally, *J. oxyphylla* presents a glandular and developed staminode, which is covered by abundant glandular trichomes secreting terpene and steroids that may participate in plant–pollinator interactions and be collected by Euglossini bees, such as *Eulaema nigrita* (Guimarães et al., 2008).

The phenolic, lipophilic, and volatile compounds revealed by histochemistry in the secretory disk of both nectarless and nectariferous flowers are among most widely distributed compounds in angiosperms (Harborne, 1997). Phenolics may have various roles in plant–pollinator interactions being part of scent, taste and color (Harborne, 1985, 1997; Nishida, 2002). Additionally, lipophilic compounds present in the nectariferous disks may enrich nectar secretion with an additional energy source (Nicolson and Thornburg, 2007), which provides a long-term metabolic fuel for pollinators (Levin et al., 2017). The presence of lipids in nectar was also referred for other *Jacaranda* species (Baker and Baker, 1975; Kram et al., 2008).

In this study, we showcase the cellular basis of nectariferous flowers with different nectar production rhythms in the same plant species. Additionally, we bring for the first time a cellular characterization of nectarless flowers' disks, which showed an unexpected production of lipophilic, phenolic and volatile substances. These changes in the functioning of the floral disk may influence plant–pollinator interaction. So, in the first and second days of anthesis, while nectariferous flowers release nectar in variable amounts and rhythms, nectarless flowers were involved in the secretion of other substances that could attract pollinators. These substances could either attract cheated nectarivores, by chemical signaling trough volatile compounds emission, or attract bees that are searching for floral resource.

FUTURE DIRECTIONS AND CONCLUSION

This study brings a broad panorama of nectarless flowers distribution and their cellular functional changes; however, we still have limitations to deeply interpret these findings in the light of plant–pollinator interactions. A question that arose from this study is if, like *Brassica rapa* (Gervasi and Schiestl, 2017), *J. oxyphylla* flowers present honest signals related to the presence of nectar, which could explain the low rates of pollinator visitation described by Guimarães et al. (2008). We should now evaluate if nectarless disks are contributing with exclusive compounds to floral scent, if the different types of flowers (early and late nectar producers, nectarless flowers) differ in their floral scents, if there is a temporal pattern of floral scent within a specific type throughout anthesis, and experimentally test the effect of the released volatile compounds on insect behavior. So, our next step is to evaluate these aspects in order to better understand the ecological implications of the cellular functional changes in secretory disks and of nectarlessness.

In conclusion, this study proposes a new paradigm, in which nectarlessness, instead of representing an energy saving strategy (Southwick, 1984; Pyke, 1991), could actually denote a higher energy investment, as the disks from nectarless flowers are producing volatile compounds instead of nectar. The little volume of nectar and the uncertainty that pollinator experience in finding a nectariferous flower, associated to the cellular functional changes in flowers' disks, paint a picture of what could be a transition from a nectar-based pollination system to another resource-based or even to a deceit mechanism of pollination in *J. oxyphylla*.

AUTHOR CONTRIBUTIONS

EG and SM contributed to the conception and design of the study. EG, PT, and SM organized the data and wrote the first draft of the manuscript. EG and PT performed the statistical analysis. LDS, LAJ, and SD performed the chemical analysis. EG, PT, and SD interpreted the results of the chemical analysis. SM performed TEM analysis. All authors contributed to manuscript revision, read and approved the submitted version.

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frontiers in Plant Science

REVIEW
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The Evolution of Sexual Fluids in Gymnosperms From Pollination Drops to Nectar

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A current synthesis of data from modern and fossil plants paints a new picture of sexual fluids, including nectar, as a foundational component of gymnosperm reproductive evolution. We review the morpho-anatomical adaptations, their accompanying secretions, and the functional compounds involved. We discuss two types of secretions: (1) those involved in fertilization fluids produced by gametophytes and archegonia of zooidogamous gymnosperms, i.e., *Ginkgo* and cycads, and (2) those involved in pollen capture mechanisms (PCMs), i.e., pollination drops. Fertilization fluids provide both liquid in which sperm swim, as well as chemotactic signals that direct sperm to the egg. Such fertilization fluids were probably found among many extinct plants such as ancient cycads and others with swimming sperm, but were subsequently lost upon the evolution of siphonogamy (direct delivery of sperm to the egg by pollen tubes), as found in modern gnetophytes, conifers, and Pinaceae. Pollination drops are discussed in terms of three major types of PCMs and the unique combinations of morphological and biochemical adaptations that define each. These include their amino acids, sugars, calcium, phosphate and proteins. The evolution of PCMs is also discussed with reference to fossil taxa. The plesiomorphic state of extant gymnosperms is a sugar-containing pollination drop functioning as a pollen capture surface, and an *in ovulo* pollen germination medium. Additionally, these drops are involved in ovule defense, and provide nectar for pollinators. Pollination drops in anemophilous groups have low sugar concentrations that are too low to provide insects with a reward. Instead, they appear to be optimized for defense and microgametophyte development. In insect-pollinated modern Gnetales a variety of tissues produce sexual fluids that bear the biochemical signature of nectar. Complete absence of fluid secretions is restricted to a few, poorly studied modern conifers, and is presumably derived. Aspects of pollination drop dynamics, e.g., regulation of secretion and retraction, are reviewed. Lastly, we discuss pollination drops' control of pollen germination. Large gaps in our current knowledge include the composition of fertilization fluids, the pollination drops of Podocarpaceae, and the overall hydrodynamics of sexual fluids in general.

Keywords: gymnosperm, nectar, nucellus, ovular secretion, pollination drops, pollen, ovule

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INTRODUCTION

Fluids play major roles during reproduction of gymnosperms. Ovule-derived fluids are almost universally found in pollen capture mechanisms (PCMs). In addition, early diverging gymnosperms are dependent on fluids for fertilization, not just for pollen capture. Before looking at the nature and complexity of these aqueous fluids it is necessary to introduce some of the aspects of reproduction that are unique to gymnosperms, beginning with pollination and then proceeding to fertilization.

A critical feature of gymnosperm pollination is that in almost all species the primary capture surface for pollen is an ovular secretion (Williams, 2009). Generally, this is called a pollination drop (Singh, 1978). Some angiosperm ovules are able to secrete fluids that influence pollen tube behavior (Franssen-Verheijen and Willemse, 1993). Ovules secrete a fluid that fills the micropyles, which attracts pollen tubes into the ovule where the pollen tube breaches the relatively thin nucellus before depositing male gametes into the embryo sac. Angiosperm ovular secretions are relatively unknown compared to pollination drops of gymnosperms. Pollination drops are a common part of extant gymnosperm pollination biology (**Figure 1**), and are found in all modern clades: *Ginkgo* (**Figure 2A**), cycads (**Figure 2B**), conifers (**Figures 2C–E**), and Gnetales (**Figures 2F–I**). These liquid-based interactions between ovule and pollen are likely to be of ancient origin. Pollination drops provide a number of conserved functions that are essential components of mechanisms involved in pollen capture, delivery, and germination. Pollination drops also provide ovule defense against microbes during reproduction (Little et al., 2014).

A distinctive aspect of some gymnosperms, and one that we will develop further in this review, is that ovules are able to secrete pollination drops that also double as attractants to pollinators. Gymnosperms that are insect-pollinated fall into two types: those that are ambophilous, i.e., the plants receive pollen by insects and wind (Meeuse et al., 1990; Kono and Tobe, 2007; Gong et al., 2016), and those that have obligate pollination mutualisms with insects, e.g., some cycads (Mound and Terry, 2001) and gnetophytes (Tang, 1987; Kato and Inoue, 1994). Although pollination drops mediate pollen capture in both types, among those that have obligate pollination mutualisms is a group of gnetalean species that reward pollinators with nectar produced by ovules (Kato et al., 1995). The evolution of nectar from pollination drops is unique to gymnosperms and will be discussed in greater depth.

In addition to fluid produced during pollination, ovules may also produce fluids during fertilization. Fertilization fluids are common to archegoniate plants, e.g., mosses, ferns and gymnosperms. These plants reproduce by means of eggs that are found inside the archegonium, the female sex organ whose presence sets gymnosperms apart from angiosperms. The structure of an archegonium is simple. A well differentiated, relatively large egg is found at the base. Above the egg, in the case of gymnosperms, is one cell; in the case of mosses and ferns, there are two cells. These cells are surrounded by neck cells, which are an adaptation to fluid-based reproduction. Upon wetting, neck cells part to allow the contents of the cells above the egg to be released. Sperm swim down this now fluid-filled passage to the egg where fertilization takes place. Whereas ferns and mosses need free water to reproduce, gymnosperms, such as *Ginkgo* and cycads, produce their own fluid. In short, reproduction with archegonia requires an aqueous medium for sperm delivery. Eventually, gymnosperm groups evolved for which this fluid requirement was bypassed.

Water is the most abundant molecule in a sexual fluid, and is important to both fertilization and to pollination in gymnosperms. However, this water is mainly a solvent for compounds that influence microgametophyte-ovule interactions. As mentioned above, early diverging embryophytes, such as mosses and ferns, are entirely dependent on water for reproduction. Since their sperm need water in which to swim it would at first appear that they do not contribute sexual fluids to this process. However, mosses and ferns release a fluid from their archegonia that is developmentally timed to assist in fertilization. When an egg ripens, the other cells within the archegonium and above the egg, i.e., neck canal cell and ventral canal cell, break down and die. The contents of these dead cells are released into the surrounding free water after the necks have separated. Contents of the dead cells further improve the chances of fertilization by creating the chemical gradients that set up sperm chemotaxis. Moss sperm were thought to be attracted to archegonia by a gradient of released sucrose (Ziegler et al., 1988). Recently, Ortiz-Ramirez et al. (2017) found that sperm chemotaxis in the moss *Physcomitrella patens* depended upon sperm ionotropic glutamate receptors. However, the specific ligand released by the archegonia that triggers this chemotactic response by the sperm remains unknown. Archegonial secretion of chemoattractants also occurs in some gymnosperms (**Figure 3**). Gymnosperms such as cycads release fluids during fertilization (Chamberlain, 1935). One such fluid is that released by megagametophyte tissues surrounding their archegonia (Takaso et al., 2013). This fluid fills the specialized fertilization chamber in which the archegonia are found (**Figure 3**). Once this chamber is filled, sperm are released from the pollen tubes and the archegonial neck cells divide forming a four-celled neck apparatus, centrally open to the egg. Archegonia release copious amounts of a white-colored substance that appears to play a role in chemotaxis (Takaso et al., 2013). Swimming sperm delivery via a microgametophyte with haustorial pollen tubes is known as zooidogamy and is characteristic of earlier diverging gymnosperms (Williams, 2009), such as *Ginkgo* and cycads. More derived gymnosperms produce gametes that are delivered by a linear pollen tube, but these gametes lack flagellae and, therefore, cannot swim. Instead, pollen tubes deliver the male gamete directly into the egg. This is called siphonogamy and occurs in all extant lineages of conifers and Gnetales. However, they sometimes still have archegonial chambers, albeit small ones, such as those found in *Picea* (Runions and Owens, 1999). The neck cells and neighboring cells surrounding the archegonium secrete lipid into the chamber space. These lipids are thought to be essential in signaling and directing pollen tubes to their destination (Runions and Owens, 1999).

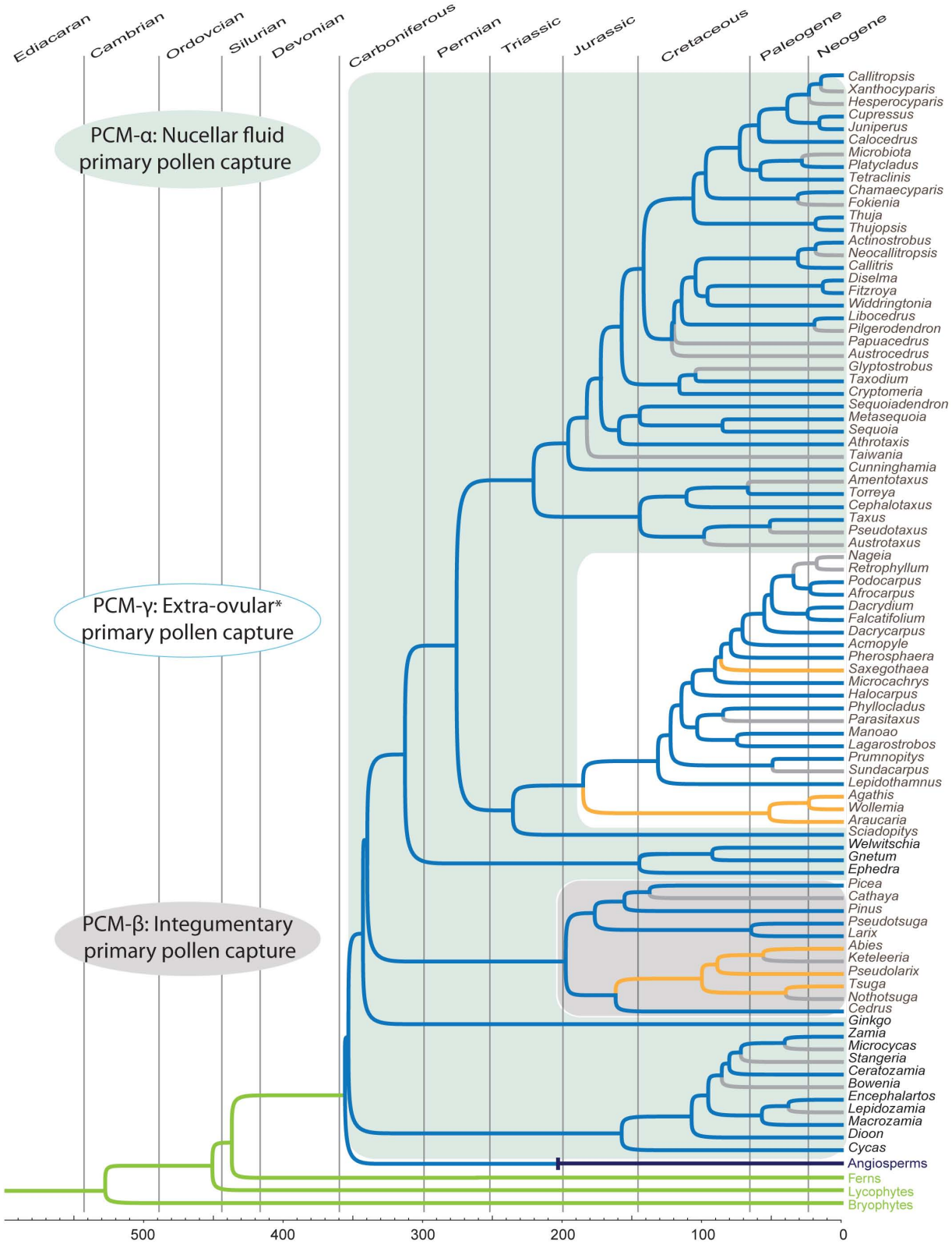

FIGURE 1 | Chronogram of the extant genera of gymnosperms based on Lu et al. (2014), and Clarke et al. (2011) for relationships, and divergence times, of angiosperms and free-sporing plants. Blue branches represent presence of pollination drops *sensu lato* (i.e., where ovular secretions from the nucellus appear

(Continued)

FIGURE 1 | Continued
between pollen capture and fertilization). Gray branches represent missing data. Yellow branches represent well-studied taxa that have been reported to lack nucellar ovular fluids in their pollination (pollination drops, *sensu lato*). Green branches represent free-sporing sex, whether homo- or heterosporous. Purple branch for angiosperms represents flower-based sex; the origin is based on one of the divergence times from Clarke et al. (2011). Light blue enclosing rectangle represent the case of the most common pollen capture mechanism among extant taxa, PCM α: nucellar fluid performing the functions of: (i) capture of non-saccate pollen, (ii) delivery of pollen into the ovule interior, (iii) germination medium of pollen, and (iv) ovule defense. Gray rectangle represents the shift to primary pollen capture by integuments in Pinaceae, PCM β. White rectangle represents the shift to various ovular, and extra-ovular primary capture mechanisms (PCM γ) in Podocarpaceae *sensu lato* and Araucariacae. Note that *Saxegothaea*, and Araucariaceae lack drops. Extinct fossil seed plants not shown; the earliest plants with seed-like structures appear in the Upper Devonian. Data for drop presence/absence from: Norén (1908), Tison (1911), Saxton (1913a,b), Doyle and Saxton (1932), Brough and Taylor (1940), Doyle (1945), Dogra (1964), Tang (1987, 1993), Takaso (1990), Tomlinson (1991, 1992, 1994), Tomlinson et al. (1991, 1997), Carafa et al. (1992), Takaso and Tomlinson (1992), Kato et al. (1995), Molloy (1995), Owens et al. (1995), Takaso and Owens (1996a, 2008), Möller et al. (2000), Mill et al. (2001), Zhuowen (2004), and Li and Huang (2006).

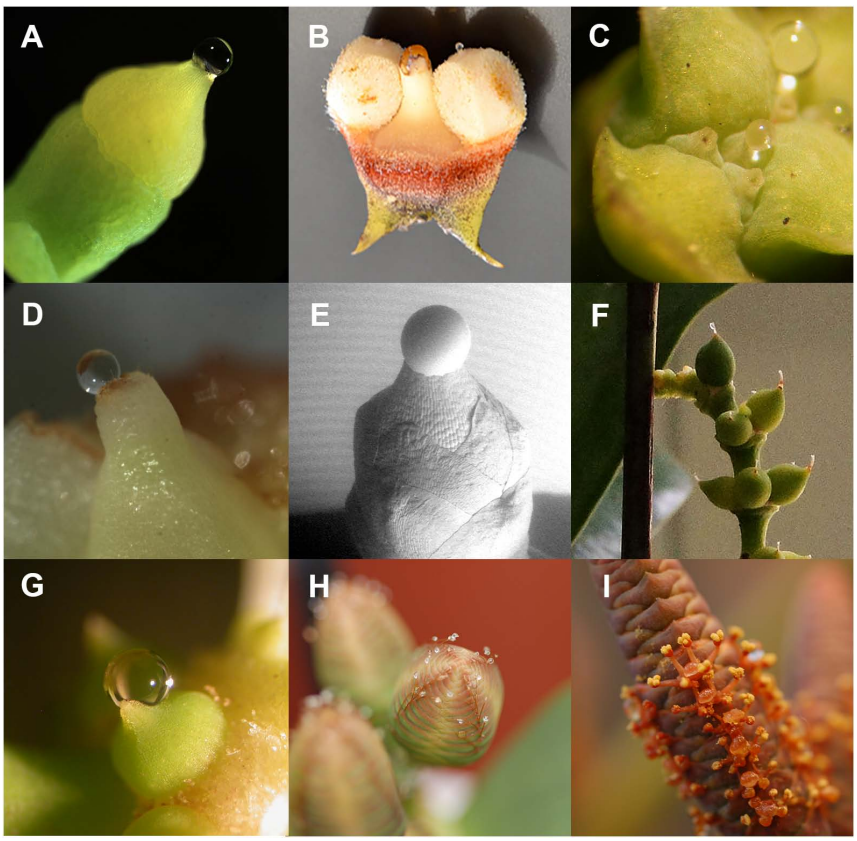

FIGURE 2 | Pollination drops **(A)** *Ginkgo biloba*, **(B)** *Ceratozamia hildae*, **(C)** *Tetraclinis articulata,* **(D)** *Pseudotsuga menziesii* (post-pollination prefertilization drop), **(E)** *Taxus* x *media* (scanning electron micrograph by A. Lunny), **(F)** *Gnetum gnemon* female, **(G)** *G. gnemon* male, **(H)** *Welwitschia mirabilis* female, and **(I)** *W. mirabilis* male.

It is the purpose of this review to trace the evolution of sexual fluids in gymnosperms, to describe the aspects of their biochemistry that we currently understand, as well as to suggest future directions of investigation. This review also has a particular emphasis, which is to trace the unique origins of gymnosperm nectar.

MODERN GYMNOSPERMS

Pollination drops are widespread among modern gymnosperms, archegonial chamber fluids less so. Pollination drops are produced by the ovule's diploid nucellus, whereas archegonial fertilization fluids are produced by the ovule's haploid gametophytes. We will discuss archegonial chamber fluids first. Although their role in sexual reproduction is clear, details of their composition are the most poorly understood of all of the gymnosperm sexual fluids.

Archegonial Chamber Fluid – Function and Composition

This fluid is mainly restricted to cycads and *Ginkgo,* the extant zooidogamous gymnosperms. Since the process of secretion takes

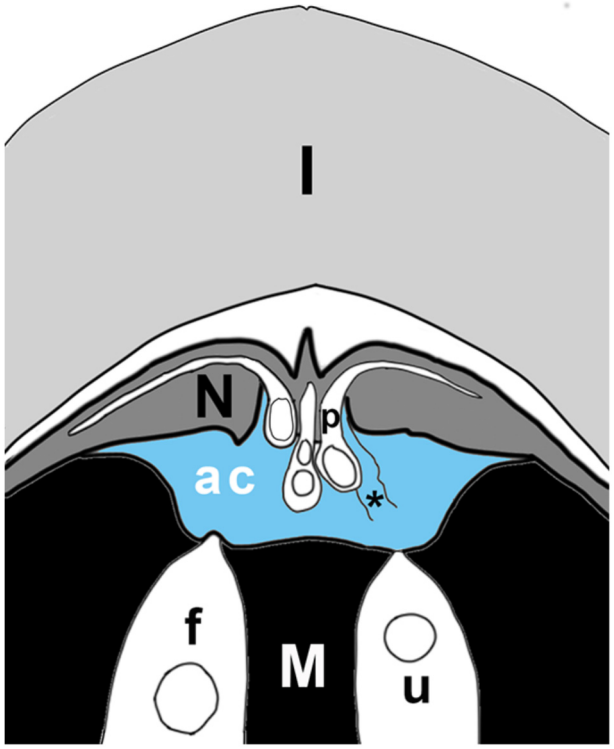

FIGURE 3 | Schematic of ovule tip at time of fertilization, showing layers of integument (I), nucellus (N), megagametophyte (M), with two archegonia (in white), of which one is fertilized (f), the other unfertilized (u). Pollen tubes (p) have grown into the nucellus; the sulcus end of the tube hangs over the archegonial chamber (ac). The archegonial chamber may be filled with fluid (blue) that originates either from ruptured pollen tubes (asterisk), from cells of the megagametophyte that line the chamber, and/or from archegonia. Some published accounts state that fluids from megagametophytes may be sufficient to fill the chamber (blue), or may be much less abundant, having only the fluids of a few ruptured pollen tubes mixed with secretions from archegonia. In the plant, the orientation of the ovule is reversed, with the megagametophyte at the top. Figure is based on Chamberlain's (1910) illustration of *Dioon edule* ovule.

place inside the ovule it is difficult to observe. Accounts of events are mostly of a descriptive, rather than experimental nature. For thorough historical discussions, see Hori and Miyamura (1997) and Norstog and Nicholls (1997). There are conflicting views as to the origins of the fluid(s). Three origins have been proposed. The first of these is the pollen tube. In *Dioon edule*, as pollen tubes rupture during sperm release, they release a fluid that is of sufficient volume (**Figure 3**) to provide a thin film in which the sperm are able to swim (Chamberlain, 1910). If pollen tubes are numerous, they may even release enough fluid to fill the entire archegonial chamber (Brough and Taylor, 1940). A second source is the megagametophyte. In *Cycas revoluta*, fluids are released from megagametophyte cells lining the archegonial chamber (**Figure 3**). Cells at the rim of the depression secrete first, followed by cells at the base (Takaso et al., 2013). A third source of fluid is from individual archegonia. In *Ginkgo biloba*, archegonial neck cells release fluid (Wang et al., 2014). Combinations of fluids are also possible, e.g., archegonial and pollen tube fluids (Chamberlain, 1910).

Some experimental work provides evidence for the functions of these fluids. In a study of pollen tubes in different conditions, Takaso et al. (2013) found that turgid pollen tubes had to be in contact with archegonial chamber fluid for a number of hours before they were able to discharge their sperm. The possibility that there may be a degree of molecular interaction between secreted pollen proteins and ovules that could be considered as a form of a recognition system was first put forward by Pettitt (1977) in his study of cycads. Pettitt's inferences were based on protein gels run from extracted whole ovules, rather than isolated fluids. He considered the context of these fluids, recognizing that the archegonial chamber fluids occur at the interface between the haploid megagametophytes and the surrounding diploid sporophytic ovule tissue. These genetically different tissues are separated from one another by a megaspore wall, which is a thick, complex structure composed of glycoproteins, cellulose, hemicellulose, and sporopollenin. The sporophyte-gametophyte *Bauplan* of the ovule imposes communication constraints (Williams, 2009). The physiological isolation that this wall imposes prevents interactions between the gametophyte and the sporophyte (Pettitt, 1979). Unfortunately, no molecular studies of protein interactions during reproduction have been carried out since these papers appeared. Even an initial analysis of archegonial chamber fluid composition has yet to be carried out. Detailed proteomic and metabolomic analysis of these fluids would add significant information to our understanding of the evolution of sperm-ovule interactions, from sperm discharge and chemotaxis through to ovule defense.

Archegonial secretions and neck canal secretions have been mainly studied by transmission electron microscopy. In both cycads with their large archegonial chambers (Takaso et al., 2013) and pinaceous conifers, e.g., Douglas-fir (Takaso and Owens, 1994) and spruce (Runions and Owens, 1999) with their small archegonial chambers, there is evidence of lipid secretion. These lipids have never been isolated and analyzed. Although collection of archegonial secretions may appear to pose sampling difficulties, with today's ultrasensitive mass spectrometers, even small samples are likely to provide results.

Pollination Drops and Related Secretions and Their Role in Pollen Capture Mechanisms

Among modern gymnosperm taxa, species have various pollination syndromes, i.e., whether they are wind pollinated and/or insect pollinated, and more specifically according to their mechanisms for collecting pollen. These mechanisms make use of secretions, i.e., lipid microdrops and/or nucellar fluids, or similar secretions. However, in a small number of species there are mechanisms that do not use secretions as far as we know (Gelbart and von Aderkas, 2002). Such mechanisms are restricted to the conifer family, Araucariaceae (Eames, 1913; Haines et al., 1984; Owens et al., 1995), and some genera of Pinaceae (Doyle and O'Leary, 1935b; Doyle and Kane, 1943), and Podocarpaceae (**Figure 1**; Doyle and O'Leary, 1935a; Tomlinson, 2012; for a

detailed review, also see Little et al., 2014). We will only touch on these mechanisms throughout; this review focuses on cases of sexual secretions and possible nectars.

Pollen capture mechanisms have been classified in several ways in the past. Traits such as pollen morphology, ovule orientation, and timing (and/or the lack) of ovular secretions have been used (Little et al., 2014). Here, we divide the modern variation known into three categories based on their primary pollen capture surface (**Figure 1**). The most widespread and ancient is PCM α (**Figure 1**; blue enclosed area), in which a nucellus-based ovular fluid extrudes from the ovule to act as the primary capture surface for pollen. This liquid surface is the first contact that pollen has with the ovule. The second major category, PCM β, has primary pollen capture by integuments, as found in Pinaceae (Doyle and O'Leary, 1935a; Doyle and Kane, 1943). Some species have a drop that appears later and brings pollen into the ovule. The third category, PCM- γ, represents pollen capture by an extra-ovular surface, typically by cone surfaces adjacent to the ovules, as observed in some Podocarpaceae (**Figure 4J**; Doyle and O'Leary, 1935b; Tomlinson, 1994).

Nectar is known only from the most prevalent type, PCM α. This nucellus-based ovular fluid also performs a myriad of functions, which include primary pollen capture, pollen delivery into the ovule, pollen germination, and defense of the ovule against pathogens.

We present a synthesis using the well-sampled genera-level phylogeny of Lu et al. (2014), and rely on their divergence time estimates to illustrate the origins and evolution of sexual fluids in extant gymnosperms (**Figure 1**). Additional divergence times and phylogenetic relationships come from Clarke et al. (2011). The presence of nucellar secretions at the pre-fertilization stage of the seed, i.e., pollination drop *sensu lato*, has been recorded and mapped on the chronogram (blue branches). The presence of the drop among modern gymnosperm clades is widespread (**Figure 1**). Regardless of possible future alternative phylogenetic hypotheses, it seems very likely that the foundational nature of ovular fluids will remain a robust inference. This is due, in part to the prevalence of sexual fluids among the majority of modern gymnosperm groups and thus the ancestral condition of having a pollination drop would be similar among all major lineages given alternative topologies (i.e., Zhong et al., 2011; Xi et al., 2013; Ruhfel et al., 2014; Wickett et al., 2014).

Pollen Capture Mechanism- α -Wind and/or Animal Pollination

In the most common PCM α (**Figure 1**), the nucellus secretes a pollination drop that first fills the micropyle, the cavity at the apex of the ovule (**Figures 4A,B**). As secretion continues, a fluid balloons outward from the opening of the micropyle in a spherical drop. In these ovules, the surfaces surrounding the opening are waxy. Hydrophobic forces between the watery secretion and the surface cause the secretion to form into a sphere. During secretion, these ovules have their micropyles facing horizontally or upward, i.e., not downward. The non-saccate pollen sinks through the drop, coming to rest inside the ovule (Tomlinson et al., 1997). By the time pollen reaches the nucellus, it is ready to germinate. The pollen tube grows and penetrates the nucellus. This mechanism occurs in *G. biloba* (Del Tredici, 2007), Cycadales (Schneider et al., 2002), Gnetales (Endress, 1996), and conifers, e.g., Cephalotaxaceae, Cupressaceae, Sciadopityaceae, Taxaceae, and the phyllocladoid species of Podocarpaceae (Tomlinson, 2012). An advantage of this PCM is that, depending on species, it serves as a key adaptation in both wind and animal pollination syndromes.

Understanding the constituents of this most prevalent PCM among extant gymnosperms is key to understanding the variety of roles that pollination fluids play in the reproductive biology of gymnosperms. We will look at water, sugars, amino acids, proteins, calcium and phosphates, as well as their role as nectar, and in pollen capture, delivery, germination, and in ovule defense.

Water

Water not only captures and hydrates pollen, but in many species, e.g., *Cupressus arizonica* (Chichiriccò et al., 2009), and *G. biloba* (Lu et al., 2016), water also causes pollen to shed its exine layer. This is an important event prior to germination. In cupressaceous conifers, exine shedding is also functionally significant. Removal of the hard-shelled exine layer, reveals the intine, which is a much more flexible layer. Additionally, exine-covered pollen is too wide to be able to enter via the ovule's micropyle, but pollen with only intine present deforms easily, allowing it to squeeze through the narrow opening (Takaso and Owens, 2008).

Isolated pollen of PCM α species generally does not germinate in water alone. Pollen of *Taxus baccata* (Anhaeusser, 1953), *Ephedra gerardiana* (Mehra, 1938), and *E. aphylla* (Moussel, 1980) readily germinated in isolated pollination drops, but did not germinate in water controls. This rules out one of the first tempting ideas about pollination drops, namely that they replace simple rainwater. We can conclude that the first of the three functions of pollination drops–pollen capture–may be largely due to water, but the other functions, germination, pathogen defense, and nectar, depend on solutes.

Sugars

The most universal and abundant solute in these watery drops is carbohydrate. The three most important sugars are glucose, fructose and sucrose. These three make up over 95% of total sugar content (TSC). In a study of sugars in pollination drops, it was found that sugars other than glucose, fructose and sucrose make up less than 1% TSC. These include melezitose and xylose, as well as two sugar alcohols (Nepi et al., 2017).

Sugars in pollination drops are necessary for pollen germination and pollen tube nutrition (Nygaard, 1977), as well as for the nutrition of insect pollinators (Kato and Inoue, 1994). When TSC is analyzed, ambophilous species can be easily separated from species that are either solely wind-pollinated or insect-pollinated. Wind-pollinated species had a significantly lower TSC than ambophilous species. TSC ranged from 20 to 50 mg/mL in the pollination drops of wind-pollinated species, whereas TSC ranged from 110 to 900 mg/mL in those of ambophilous species (Nepi et al., 2017).

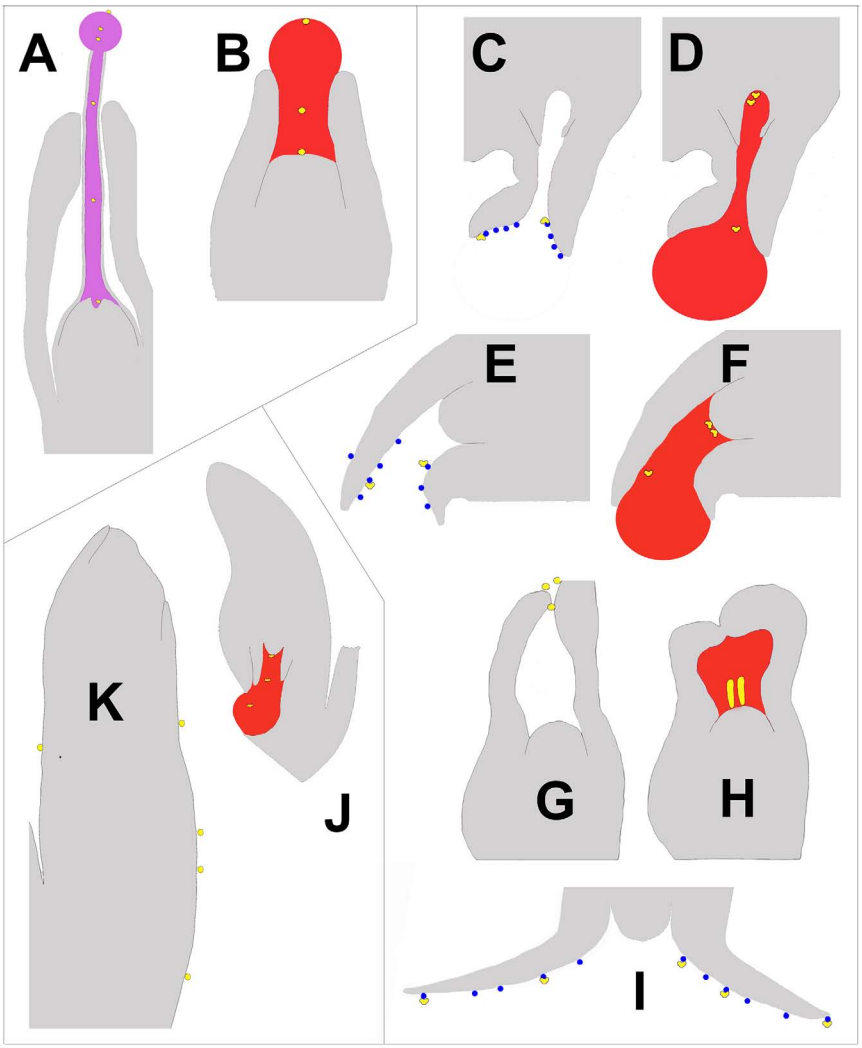

FIGURE 4 | Schematic of longitudinal sections of portions of ovules at time of pollination illustrating the three types of pollen capture arranged clockwise. Nectar is pink, pollination drops are red, and lipid microdrops are blue. Pollen is yellow and is either round or saccate, depending on the mechanism. **(A)** *E. foeminea* – PCM α (after Rydin et al., 2010). The lowest pollen grain can be seen entering a depression in the nucellus known as the pollen chamber, which is formed by PCD. **(B)** *Taxus canadensis* – PCM α (after Dupler, 1920), **(C,D)** *Picea sitchensis* – PCM β (after Owens and Blake, 1985). The two uppermost pollen grains can be seen floating into a pollen chamber. **(E,F)** *Cedrus deodora* – PCM β (after Takaso and Owens, 1995a,b). **(G,H)** *Larix decidua* – PCM β (modified from Doyle, 1945). **(I)** *Abies amabilis* – PCM β (modified after Chandler and Owens, 2004). **(J)** *Podocarpus* – PCM γ (after Doyle, 1945). **(K)**. *Agathis australis* – PCM γ (after Owens et al., 1995).

The universality of sugars in pollination drops implies that they were present among the ancestors of extant gymnosperms. Although analyses tend to report stable sugar compositions, in some species of *Gnetum*, sugar concentration can vary according to relative humidity. This is due to the high relative water content of the surrounding atmosphere, e.g., measurements of TSC of pollination drops of *G. gnemon* growing in a tropical rainforest ranged from 3 to 13% over the course of an evening (Kato et al., 1995).

Amino acids

All pollination drops have amino acids (Chesnoy, 1993). These include serine, glutamic acid, glycine, histidine, alanine and proline (Nepi et al., 2017). Just as sugar concentrations can be used to discriminate pollination drops of wind pollinated species from those of ambophilous species, the total amino acid content (TAC) of drops also proves to be a reliable predictor of pollination syndromes. Wind pollinated species have higher TAC values than ambophilous species such as *Gnetum gnemon*. From a nectar standpoint, it is not just a low total TAC that is important, but among the low concentration amino acids the relative concentrations of certain types of amino acids are significant also. One class of amino acids–non-protein amino acids–is characteristic of nectar. β-alanine, for example, may have desirable neurophysiological effects on insects that reinforce the role of nectar in attracting insects (Nepi et al., 2017). Concentrations of γ-aminobutyric acid, a suspected neurostimulant of insects, are very low if not zero

in wind-pollinated gymnosperms, such as *Cephalotaxus* spp. (Chesnoy, 1993; Nepi et al., 2017).

Proteins

Proteins are found in all gymnosperm sexual fluids that have been analyzed to date. Because proteins are large complex molecules, by definition, they represent a sporophytic investment in the pollination drop that is substantial. This would be the case in what can be described as the "secretome," i.e., proteins processed and secreted into the pollination drop by a tissue such as the nucellus. These proteins are thought to be active in the apoplast. However, some proteins are found in pollination drops as a consequence of cellular breakdown and are not normally found in the apoplast. This "degradome" is a consequence of nucellus cell death/breakdown to form a pollen chamber, for example in *Ephedra* spp. The degradome can be composed of over a dozen proteins (von Aderkas et al., 2015).

The most common proteins of the secretome include carbohydrate-modifying enzymes, such as glucanases, and defense proteins, such as anti-fungal enzymes, e.g., thaumatin-like proteins. These classes of proteins are nearly universal in pollination drops, which implies that they may have been there since the beginning of gymnosperm reproduction. As such, they represent a relatively well-preserved fraction of the functions of the pollination drop (Wagner et al., 2007).

Recently, proteomic analysis of pollination drops, coupled to a transcriptomic analysis of nucellus, was carried out on *Cephalotaxus koreana* and *C. sinensis* (Pirone-Davies et al., 2016). Pollination drops of these species have rich secretomes with nearly 30 proteins, many of which are involved in defense, carbohydrate-modification, or pollen growth. There are also a number of unique proteins that likely function in starch and callose degradation. This parallel gene expression study revealed a number of transcripts likely involved in pollination drop secretion, such as sugar transporters, β-glucosidases and P-loop-containing nucleoside triphosphate hydrolases.

In addition to such carbohydrate-modification and defense-related proteins just described, proteins have also been found that may play a role in regulating pollen growth and selection. Arabinogalactan proteins were found in pollination drops (O'Leary et al., 2004), which are involved in sporophytic selection of pollen tubes in angiosperms.

Protein composition of pollination drops of cycads, *Ginkgo* and many groups of conifers have yet to be studied. In addition, protein profiles, comparing male and female nectars found in strobili of the Gnetales need to be analyzed, as they may show differences as seen in angiosperms (Chatt et al., 2018).

Calcium and phosphate

Calcium is important for pollen germination (Brewbaker and Kwack, 1963). Recent studies have shown it to be present in *Ginkgo* pollen intine (Lu et al., 2016). Phosphate was identified long ago in pollination drops of *T. baccata* and *E. distachya* (Ziegler, 1959), but the form of phosphate was not established. Recently, we found evidence in a transcriptomic analysis of *Cephalotaxus* nucellus during pollination drop secretion of expression of a gene involved in eATP regulation – an apyrase (Pirone-Davies et al., 2016). Since phosphates, such as extracellular ATP (eATP), have immunogenic functions, including regulation of responses to fungal invasion in seed plants (Gust et al., 2017), pollination drops ought to be analyzed for their phosphate content.

Overall patterns in PCM α of nectariferous vs. non-nectariferous pollination drops

The strongest evidence that differentiates nectar from non-nectar pollination drops comes from the recent Principal Component Analysis (PCA) of carbohydrates and amino acids of ovular fluids (Nepi et al., 2017). PCA effectively separates out ambophilous from wind-pollinated species. The main factors in the clustering of the samples were; TSC (low in anemophilous species; high in ambophilous species), TAC (high in anemophilous species; low in ambophilous species), and non-protein amino acid percentage (low/absent in anemophilous species; high in ambophilous species). Absolute concentrations explained 70% of the variation. Ambophilous species overlap with flowering plant nectar (Nepi et al., 2017). In the PCA analysis, cycads, such as *Zamia furfuracea* that are beetle-pollinated (Norstog et al., 1986), clustered closer to the wind-pollinated conifer species, because of the low concentrations of sugar in their drops. However, a significant percentage the amino acids present was that of β-alanine, a rewarding compound for insects. Nepi et al. (2017) concluded that natural selection for strictly nutritional needs of these insects had had a lower impact on the chemistry of these cycad pollination drops. Chemical analysis also yielded a surprise: profiles of *G. biloba* pollination drops firmly placed this species among ambophilous species, namely those species for which nectar was a significant reward to insects. *G. biloba* is often referred to as wind-pollinated, e.g., Jin et al. (2012a), but as it is the last remaining species of what was once a species-rich clade, the PCA analysis would suggest that not just the surviving species of *Ginkgo*, with its high sugar concentration and non-protein amino acids, was once or still is, insect-pollinated, but that extinct ginkgophytes may have also been insect-pollinated.

What are the differences between a PCM α pollination drop and nectar? In our opinion, there are not many. In terms of evolution, the original pollination drop of the common ancestor of seed plants must have had at least the same four functions seen in extant species with PCM α: microgametophyte capture, delivery, germination, and ovule defense. Later, or possibly very early on, this drop acquired another function – insect reward. Such a pollination drop can be called either nectar or a pollination drop with a nectar function (Jörgensen and Rydin, 2015), but it is more expedient to focus on the ecological services, and call it nectar. The diversity of modern nectar types has resulted in nectar terminology being beset by historical circumstance (for discussion see Koptur, 1992). For example, angiosperm nectaries were the first to be divided into floral and extra-floral nectaries (EFNs), which has led to fern nectaries being referred to as EFNs, since they lack flowers. As Marazzi et al. (2013) point out in their survey of nectar-producing tissues, almost every above-ground part of flowering plants has been associated with nectar production. Nectar secretion processes are diverse enough to defy simple categorization based on anatomy. Nectar, it turns out,

does not always flow from a nectary. Nectar is simply a sweet apoplastic fluid available on a plant surface where it can attract some animal or other that consumes it as a reward. Like many fern and angiosperm nectars, gymnosperm nectar does not, in the case of PCM α, originate from a nectary. Since it is of uniquely ovular origin, there is probably no modern angiosperm homolog. The nectar definition resides on the ecological service provided, that is, the mutualism of which it is a part. Nectar secreted by ovules of gymnosperms attracts many pollinators such as lizards (Celedón-Neghme et al., 2016), nocturnal moths (Kato and Inoue, 1994; Rydin and Bolinder, 2015), flies and wasps (Kato et al., 1995; Wetschnig and Depisch, 1999), even ants (Bolinder et al., 2016). It is the considered view of some nectar experts that pollination drops are functionally equivalent to angiosperm nectar (Bernardello, 2007).

Nectar production, when it is well-known, occurs in extant gymnosperms with PCM α, and thus far appears to be restricted to dioecious species. *Gnetum* spp., *E. foeminea*, and *Welwitschia mirabilis,* produce nectar from both male and female strobili (Nepi et al., 2017). In contrast, wind-pollinated species of *Ephedra* lack nectar production on their male strobili (Bolinder et al., 2016). In both the female and male strobili of *Gnetum* and *Welwitschia*, ovules produce drops that are sugar-rich and contain non-protein amino acids (Nepi et al., 2017). The largest difference between males and females is that the ovules in the male strobili are non-functional, sterile structures, the only function of which appears to be secretion (Haycraft and Carmichael, 2001). This is one of the unique aspects of nectar production among extant gymnosperms. It would be interesting to investigate gene regulation of ovule development to see whether ovules in male strobili are indeed different from those in female strobili. Because turning an ovule to another purpose is not common among plants, it would be of interest to know whether ovule development is redirected only for the purpose of providing nectar to attract insects. Nepi et al. (2017) found that male nectar had less volume, with lower TSC than female nectar. Compositional differences also exist. Fertile ovule secretions had greater fructose concentrations than those of male secretions. Higher concentrations of non-protein amino acids were found in fertile ovules than in male secretions. This is similar to results reported for male and female flowers of flowering plants. For example, in species of *Cucurbita*, male and female flowers of *Cucurbita maxima* ssp. *andreana* differ in their overall nectar production (Ashworth and Galetto, 2002), *C. pepo* male and female nectars differ in their sugar composition (Nepi et al., 2001), and *C. maxima* cv. Big Max male and female nectars differ in both metabolome and proteome (Chatt et al., 2018).

Nectar in male plants has two possible sources. The first source is pollination drops of the PCM α type, which produce a nectar in *G. parviflorum,* for example, which moths will search out with their probing proboscises (Kato et al., 1995). Moths will also search for any nectar that has seeped onto collars (Rydin et al., 2010). In some ways the situation is analogous to EFNs of plants such as *Acacia longifolia*, in which EFNs are in very close proximity to floral organs, which lack nectaries. Birds seeking nectar from EFNs unavoidably pollinate the flower (Thorp and Sugden, 1990). A second and more controversial nectar source in gymnosperms has been reported from male plants of *E. aphylla* (Bino et al., 1984). Here, nectar is non-ovulate in origin: it is produced from epidermal stomata of bracts of male cones. Although there are micrographs showing stomata and the sub-epidermal tissue of this nectary, the function of these nectaries has been called into question (Bolinder et al., 2016) and ought to be more closely investigated, as it is the only case of non-ovular nectar source known in any extant gymnosperm.

Once nectar is invoked, it raises several questions. There are a range of insect behaviors that must be considered. Is a pollination drop still nectar if it only occasionally feeds opportunistic insects, only minimally contributing to reproductive success? Opportunistic nectar feeding by a broad range of insects, including those that are not considered pollinators (i.e., ants), has been described by various authors (Little et al., 2014; Bolinder et al., 2016). Another question concerns whether a pollination drop is still nectar if it attracts parasitic insects that do not contribute at all to the reproductive success of the plant. Chalcid wasps that parasitize ovules are attracted to pollination drops of *Ephedra* (Moussel, 1980; Bolinder et al., 2016). In addition to parasitizing the ovules, these wasps feed on pollination drops also. Furthermore, the wasps can be present in sufficient numbers that they consume the majority of drops produced by ovules in the local plant populations. After sucking up the pollination drops, the insects oviposit their eggs into the ovule (Moussel, 1980). At first glance, one would expect that a seed parasite such as a chalcid wasp would be ruining its own opportunities by depressing the plant's ability to set seed, but these parasites are able to alter megagametophyte metabolism in such a way that the ovule – in spite of its reproductive failure – fills with the very reserves its embryo would require, only now they are solely available to the parasite (Favre-Duchartre, 1960). In this case, the nectar is only the first in a series of high energy substances that the parasite uses for its own offspring. In this, nectar-producing gymnosperms are victims just as much as non-nectar producing gymnosperms. For example, the chalcid wasp *Megastigmus spermotrophus,* a seed predator, parasitizes megagametophytes of *Pseudotsuga menziesii*. By injecting venoms, the chalcid may be redirecting the megagametophyte's metabolism (Paulson et al., 2016).

There are other aspects of nectar that await study in gymnosperms. For example, if we look to angiosperm nectar, a diversity of secondary metabolites has been found that affect the interactions between plants and their pollinators (Roy et al., 2017). In gymnosperms, analyses are lacking for a number of classes of metabolites, including lipids, phenolics and terpenoids that might be present in gymnosperm nectar. Another aspect of gymnosperm nectar that warrants at least preliminary study is a possible nectar microbiome. For example, a number of angiosperm species have been discovered harboring yeasts in their nectar (Nepi, 2017). It is not unreasonable to expect a microbiome in these nectars that are exposed to the environment and have complex plant-animal interactions.

Pollen Capture Mechanisms β and γ

The remaining PCMs differentiate themselves from PCM α in morphology, behavior and chemistry. In our simplified

classification of PCMs, we present two other basic types of PCMs, β and γ that relate to both primary pollen capture surface and clade. PCM β represents a diverse set of pollination mechanisms found in Pinaceae in which the primary pollen capture surface is the integument. Many of these use lipid-based microdrops as part of this primary capture (**Figures 4C–I**). The clade comprised of Podocarpaceae and Araucariaceae (**Figure 1**) possesses PCM γ (**Figures 4J,K**). Generally, in these, extra-ovular surfaces capture pollen. Some phyllocladoid podocarps use drops for pollen capture similar to PCM α, but in these cases the pollen either lack sacci or have vestigial/non-functional sacci (Tomlinson, 2012). This is an interesting parallel with one pinaceous species, *Picea orientalis*, in which sacci have become non-hydrodynamic and ovules remain upright at time of pollen receptivity (Runions et al., 1999). Currently, it is thought that all species with PCM β and PCM γ are anemophilous. However, there is compelling recent evidence that ancestors or extinct sister-groups of these clades may have been ambophilous in the Mesozoic (Labandeira et al., 2007; Ren et al., 2009; Labandeira, 2010). Consequently, the biochemical profiles of these PCMs are of immediate importance in any discussion of ancient nectar production in a clade that has seemingly lost that capacity.

There are a number of ways in which PCMs β and γ differ from PCM α. In PCM β, found among Pinaceae (e.g., *Picea*, *Pinus*), lipid microdrops are secreted from two integument extensions, or flaps, at the tip of the ovule. Pollen adheres to these microdrops. An ovular pollination drop is then produced, which removes the pollen from the integuments. Since the pollen of these species is saccate, a morphological feature that confers buoyancy, the pollen floats upward into the ovules (Owens et al., 1987). There is a selective element to this, as saccate pollen is preferentially taken up compared to non-saccate pollen (Leslie, 2010). The ovules of species with saccate pollen are characteristically inverted at the time of pollination. This is in contrast to PCM α where non-saccate pollen sinks into the drops of more or less upright ovules. A variant of the pinaceous PCM β seen in *Cedrus* has pollen captured by microdrops on an irregular funnel shaped integumentary margin, with a drop arriving later to deliver pollen into the ovule (Saxton, 1930; Takaso and Owens, 1995a,b). *Pseudotsuga* and *Larix,* have non-saccate pollen that is trapped by sticky, terminal integumentary hairs. These hairs collapse inward, which physically delivers the pollen into the ovule interior. Many weeks or a few months later a drop is secreted (Takaso et al., 1995) that brings the pollen to the nucellus surface (Takaso and Owens, 1997), and germination is triggered (Villar et al., 1984; Said et al., 1991). This drop has been called a post-pollination prefertilization drop. It is smaller than a PCM α pollination drop and fills the volume of the micropyle only (**Figures 4G,H**). *Abies* appears morphologically similar to *Cedrus*, but is thought to lack a pollination drop (Owens and Molder, 1977; Chandler and Owens, 2004).

In the case of PCM γ, which is restricted to Podocarpaceae (Tomlinson, 1991, 1994), neither the tips of the micropyle, nor the surrounding surfaces of the bract are coated with wax. Subsequently, the pollination drop is not hemispheric, but assumes a spreading amorphous form that scavenges pollen from a larger area than is possible with PCM α (drop capture) or PCM β (integumentary capture). In some species, an ovule may repeatedly secrete and withdraw its pollination drops. Similar to the pinaceous PCM β, pollen are saccate, and ovules inverted. Again, pollen entry into the ovule is due to flotation. However, there are no known surfaces with lipid microdrops (i.e., PCM β) as part of primary pollen capture in PCM γ.

Sugars

Analysis of pollination drops from species with PCMs β and γ has not been done in any broadly sampled, systematic way. However, carbohydrate concentrations have been reported from pollination drops of several taxa of the pinaceous PCM β, including *P. engelmannii* (Owens et al., 1987), *Pinus nigra*, and *P. resinosa* (McWilliam, 1959), which all have low TSC, i.e., concentrations are generally less than 5%. Among sugars, fructose dominates: there is little glucose and generally no sucrose. Polysaccharides such as galactose, arabinose, rhamnose and mannose are often detected, but at low concentrations (Chesnoy, 1993). Drops of *Pseudotsuga menziesii* also have similarly low concentrations of these carbohydrates, whereas *Larix* x *marschlinsii*, (in the genus sister to *Pseudotsuga*), has a relatively high concentration of sucrose, e.g., 53 mg/mL (Nepi et al., 2017). Differences in the TSC between these species is thought to be responsible for the differential responses of pollen that were observed after application of cross-generic pollen (von Aderkas et al., 2012). No sugar concentrations from species with PCM γ are as yet known.

Amino acids

The profiles of amino acids in PCMs β and γ, where known, are typical of wind-pollinated species. Amino acids include serine, aspartic acid, glutamate, proline, glycine, α-alanine, and traces of others, such as leucine, isoleucine, threonine, glutamine, aspartate. Non-protein amino acids, such as β-alanine that are present in nectar of all ambophilous species with PCM α, are almost completely lacking in taxa that have PCMs β and γ (McWilliam, 1959; Nepi et al., 2017).

Lipids

Lipids also appear commonly as microdrops on integumentary extensions of *Picea* (Owens et al., 1987), *Pinus* (Owens et al., 2001), and *Cedrus* (Takaso and Owens, 1995a). Unfortunately, no chemical analyses of these integumentary lipid secretions have been made to date.

Proteins

The only species that have been studied outside of PCM α are *L.* x *marschlinsii* and *P. menziesii* (PCM β). Just as in PCM α, defense proteins such as chitinases (Coulter et al., 2012) and thaumatin-like proteins (O'Leary et al., 2007) were identified. In addition, the *in situ* activity of these enzymes has been confirmed. Carbohydrate-modifying enzymes have also been found, including xylosidases, galactosidases (Poulis et al., 2005), and invertases (von Aderkas et al., 2012). Serine carboxypeptidase, peroxidase, and aspartyl protease were detected (Poulis et al., 2005). In summary, many of the

same enzymes involved in ovule defense and carbohydrate-modification are found across all gymnosperms, implying a conserved ancestral function. However, this conclusion is based on two species and begs further investigation of PCM β. Species of PCM γ remain unsampled for proteins.

Calcium and phosphates

Calcium is abundant in post-pollination prefertilization drops of *Larix* and *Pseudotsuga* (von Aderkas et al., 2012). Phosphate compounds await investigation. Again, this is likely to be conserved among gymnosperms, but further study is needed for confirmation. Given that both of these compounds are well-known and important in angiosperm pollination biology, investigation of these compounds represents a key gap in our knowledge.

Overall patterns in PCM β and γ of nectariferous v non-nectariferous drops

Species with PCMs β and γ are not involved in nectar production today, but according to Ren et al. (2009) ancient members of these might have had insect pollinators. In particular, PCM β has been discussed in relation to insect pollination in the Mesozoic (Ren et al., 2009). Given that today there are no such insect-pollination drop relations among extant species with PCM β, how is this possible? An interesting possibility is already available in the case of species such as *L.* x *marschlinsii*, which has higher sugar concentrations (∼100 mg/mL) compared with other conifers. The recent analyses of Nepi et al. (2017) adds support for the idea that in the Mid-Mesozoic there may have been conifers that produced a passable nectar and could have been insect-pollinated, specifically by long-proboscid scorpion flies (Peris et al., 2017). This implies that a trait such as total sugar concentration (TSC) in pollination drops may be under natural selection, and as a result, insect pollination mutualisms are more likely than previously thought. It is certainly within the realm of possibilities, because recent phylogenetic analysis of *Ephedra* provides evidence that in at least one gymnosperm clade pollination syndromes evolved from the plesiomorphic state of insect-pollination to wind pollination (Bolinder et al., 2016). If it could have happened in the gnetalean *Ephedra*, could it also have occurred in ancient Pinaceae? For example, similar reversion from insect to wind pollination is common in angiosperm species, where it has occurred as many as 60 times (Koptur, 1992). More *direct* observation of insect pollinators is required. Because insect pollinator communities thrive in ecosystems that provide resource diversity, as pointed out in Saunders' (2018) meta-analysis of insect pollinators collecting pollen from wind-pollinated plants (including Pinaceae), it is not surprising that even a little bit of carbohydrate-supplemented fluid probably goes a longer way in attracting insects than previously thought. Given that modern insects visit anemophilous species for pollen nutrition (Saunders, 2018), and that simple changes in regulation of invertase gene expression genes, as is also known to occur in flowering plants, results in changes in sugar concentration (Heil, 2015), we suppose that shifts from insect to wind pollination in gymnosperms may be more likely than previously thought.

FOSSIL GYMNOSPERMS

Integrating information from modern gymnosperm ovular fluids with the fossil record presents a challenge. To further our understanding of the origins of sexual fluids in seed-plants we must rely on a synthesis of data from modern plants with inferences based on morphological and anatomical fingerprints of biological function in the context of current phylogenetic hypotheses. By way of example, the earliest cycads, the crown group of which dates back to the mid-Permian (265 Mya) (Condamine et al., 2015), likely reproduced in a manner identical to how they reproduce today. Their conserved ovular features imply as much, even though direct fossil evidence of sexual fluids may be lacking. Direct observation of sexual fluids is expected to be rare precisely because of their ephemeral nature, but not impossible. Certain fossil localities with exceptional preservation (Lagerstätten) have shown rare cases of preserved plant exudates, e.g., mucilaginous plugs in the aroid seed, *Keratosperma,* from the Eocene Princeton Chert locality (Smith and Stockey, 2003). A permineralized pollination drop that contains prepollen is known from a callistophytalean from the Carboniferous (Rothwell, 1977). Much of the fossil evidence supporting a long history of sexual fluids is not based on direct discovery of preserved pollination drops, but on sound inferences made from anatomical fingerprints related to gymnosperm reproduction (Stewart and Rothwell, 1993).

Timeframe

The earliest fossil records for gymnosperm reproduction date from the Devonian. A megasporangium/nucellus (**Figure 5**) is surrounded by axes, or laminar organs, borne on structures called cupules (Stewart and Rothwell, 1993). Homologies drawn between modern ovules and these preovules have been the source of much discussion in paleobotanical studies (Taylor and Millay, 1979; Leisman and Roth, 1984; Meyen, 1984; Hilton and Bateman, 2006). Generally, the structures surrounding the megasporangium have been called integumentary lobes, because they are considered to be homologous to the single gymnosperm integument (Taylor et al., 2009). The retention of a megasporangium on the sporophyte is called the seed-habit, which is defined, at least in part, by whether embryos mature on the sporophyte, and by where integuments form the micropyle (Herr, 1995).

Supporting arguments for ancient origins of sexual fluids come from studies of microgametophytes (prepollen and pollen), and both preovules and ovules. The presence of liquids is often implied if a structure described from fossils is similar to a fluid secreting/vectoring structure known to function during reproduction. The examples provided below include prepollen, sperm, the functional requirements of saccate pollen, and the adaptations for pollen and/or sperm delivery such as channels for fluid-based pollen delivery and signatures of fluid production, such as cellular break-down in pollen chambers. At the end of this section we will also touch on the kind of fossil evidence for nectar.

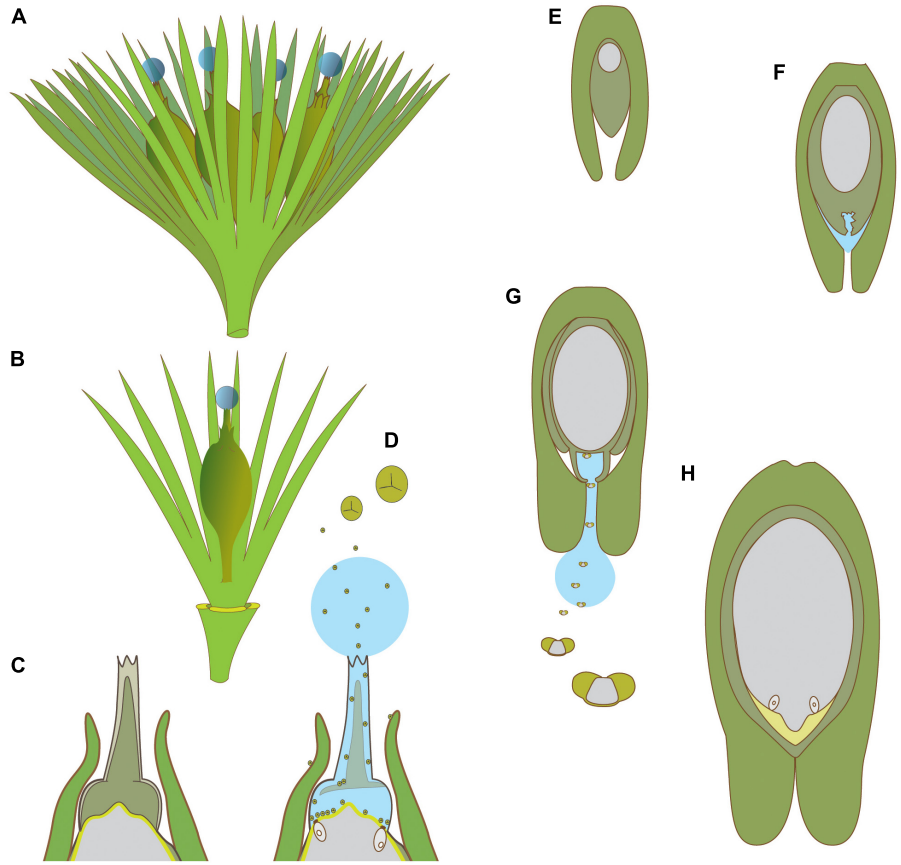

FIGURE 5 | Schematic diagrams of pollination in hydrasperman ovules that appeared in the Devonian **(A–D)** and callistophytalean ovules that appeared in the Pennsylvanian **(E–H)**. **(A)** Cupule bearing four ovules with pollination drops. **(B)** Cupule quarter showing one ovule; three other cupule segments removed. **(C)** Longitudinal section through apex of ovule prior to pollination; megagametophyte (center, light gray) is developed, apical region of nucellus (dark gray) with tissue filling the salpinx (central column). **(D)** Longitudinal section through apex of ovule at time of pollination; central column tissue degrading (inferred PCD, see **Figures 4A,C,D** for examples in extant species), pollination drop present with trilete prepollen falling through the pollination drop; megagametophyte and archegonia exposed to prepollen. **(E)** *Callospermarion* (Callistophytales) ovule showing early stage with developing megagametophyte (center, gray) within the nucellus (dark gray). **(F)** Later stage with nucellus beginning to break down (inferred PCD), and pollination drop is forming. **(G)** Pollination stage with well-developed pollen chamber; pollination drop collecting saccate *Vessicaspora*-type saccate pollen grains that float up the drop to the nucellus surface where they germinate to produce haustorial pollen tubes, similar to those seen in extant cycads. **(H)** Fertilization stage with archegonia formed; apical chamber filled with fertilization fluid for swimming sperm (microgametophytes not shown). **(A–D)** based on Matten et al. (1980, 1984), Rothwell and Wight (1989), Serbet and Rothwell (1992), Erwin et al. (1994), Hilton and Bateman (2006), Galtier et al. (2007), Prestianni and Gerrienne (2015). **(E–H)** based on Rothwell (1971), Rothwell (1977).

Prepollen

Prepollen characterizes virtually all Paleozoic gymnosperms (Poort et al., 1996). Having a proximal aperture similar to that found in modern free-sporing heterosporous plants is an indicator of endosporic microgametophyte development and release of swimming sperm from the aperture at maturity (Chaloner, 1970; Rénault, 1887). Prepollen is thought to have germinated proximally, via the monolete or trilete meiotic groove as in free-sporing plants. Observation of ephemeral free swimming sperm in fossils is understandably rare (but see Benson, 1908; Stewart, 1951; Nishida et al., 2003). The transition from prepollen to modern pollen has been studied, although it is not clear for all groups, for example, Cordaitales and voltzialean conifers have either prepollen, or modern-looking pollen with a distal aperture, and in some instances both (Gomankov, 2009). It is possible that many extinct taxa had a transitional type of microgametophyte development between prepollen and modern siphonogamous pollen, similar to that seen in cycads and *Ginkgo* in which the pollen tube germinates distally to produce haustorial tubes, penetrates the nucellus, and develops later to release swimming sperm proximally. Haustorial pollen tubes have been observed in Callistophytales (Rothwell, 1977; **Figures 5E–H**). Preserved spermatozooids within microgametophytes inside the apices of ovules have been documented for a glossopterid (Nishida et al., 2003, 2004, 2007). Taken together, the presence of prepollen allows us to infer the presence of archegonial fertilization fluids. This is further supported by the preserved archegonial chambers – the site of sperm delivery – in the ovules of seed ferns such as *Lagenostoma* (Taylor and Millay, 1979).

Hydrasperman Anatomy

Modern gymnosperms and early seed plants have similar-looking pollen delivery channels. In modern gymnosperms the micropyle is formed by the integument; this tube is the pathway for sexual fluids for the direct or secondary capture of pollen, which later germinates to produce pollen tubes that penetrate the nucellus (see section above on extant PCMs α, β, and γ). In the earliest seed plants, there is a micropyle analog, formed from the apex of the megasporangium, or nucellus, called a salpinx (**Figures 5C,D**; Matten et al., 1980, 1984; Rothwell and Wight, 1989). Hydrasperman prepollen-receiving anatomical structures have been interpreted and labeled in different ways (see discussion by Hilton and Bateman, 2006). Prepollen is found within the salpinx in anatomically preserved fossils (Matten et al., 1980). Seeds appearing later in the fossil record maintained a modified version of this hydrasperman apical modification, including the members of the Lyginopteridales and the Medullosales (Meyen, 1984; Doyle, 2008). Thus, one of the interpretations is that similar shapes used by modern gymnosperms for pollen capture by a sexual fluid, i.e., a PCM α-type drop, were probably present in these extinct plants.

Saccate Pollen Grains

In modern gymnosperms, saccate pollen are a hydrodynamic adaptation in which the hydrophobic nature of the pollen wall allows the pollen grain sacci to inflate upon contact with the pollination drop (Salter et al., 2002). Sacci and inverted ovules are another anatomical fingerprint for drop delivery at the time of pollen receptivity. Sacci provide buoyancy for the grain, which is then able to float upward in the drop through the micropyle to the nucellus, where the pollen germinates (Doyle, 2008; Leslie, 2008, 2010). A notable example from the fossil record is in the saccate glossopterids (Nishida et al., 2003, 2004, 2007). Their cycad-like microgametophytes, which have been found preserved in the apex of fossil seeds, have mature sperm cells just prior to release. Among conifers, the developmental link between saccate pollen and pollination drops is of considerable importance in the evolution of conifer pollination mechanisms (Leslie et al., 2015). It is interesting to note that saccate pollen is prevalent among many extinct gymnosperms lineages, including Peltasperms, Corystosperms, Callistophytales, Cordaites and Voltizales *sensu lato* (Doyle, 2010; Bomfleur et al., 2013).

Nucellar Degradation, Pollen Chambers and Micropyles

In many gymnosperms, pollination drop secretion coincides with breakdown of apical nucellar tissue (Singh, 1978), presumably by programmed cell death (PCD). In cycads, *Ginkgo*, Gnetales, and some Pinaceae, cells degrade to form a chamber (**Figures 4A,C,D**). Protein profiles of these drops show the expected signature of a degradome that is predicted for a PCD-derived exudate (von Aderkas et al., 2015). Virtually all Paleozoic fossil ovules, e.g., hydraspermans, Lyginopteridales, Medullosales, that are anatomically preserved show some degree of apical nucellar cellular breakdown to form (pre)pollen chambers. The earliest seeds with anatomical preservation show signs of PCD during pollination (**Figures 5C,D,F**; Rothwell, 1971; Matten et al., 1980). Signs of PCD in fossil nucellar apices provides another anatomical fingerprint for the presence of pollination drops.

Presence of Prepollen and Pollen in Pollen Chambers

It is unlikely that significant numbers of prepollen or pollen could accumulate by chance and gravity alone into the pollen chambers of ovules. As in modern gymnosperms, some mechanism must have existed to increase efficiency. In modern gymnosperms, the drop captures directly (PCM α) or scavenges secondarily from integumentary (PCM β) or extra-ovular surfaces (PCM γ) to bring pollen into the interior of the ovule (Tomlinson et al., 1997). In hydraspermans, prepollen grains are often found in anatomically preserved ovules (Taylor et al., 2009). Where integumentary lobes are short, i.e., around the ovule, salpinxes are reduced. This suggests the extension of the salpinx, a structure for capturing pollen, is to optimize exposure of the drop to the environment for prepollen capture. Niklas (1983, 1985) shows that preovules with integumentary lobes close to the salpinx had greater numbers of simulated prepollen capture events, although this includes several other factors, such as orientation of the preovules. The long micropyles with pollination drops of modern-day Gnetales function similarly to capture pollen (El-Ghazaly et al., 1998). Fluted, tubular, apical micropylar structures bearing pollen grains in their base are common in anatomically preserved fossil ovules. It has been argued that increasing the distance that microgametophytes and their gametes have to travel to achieve fertilization represents a trend of increasing sporophytic control of microgametophyte development (Lora et al., 2016).

Nectar

Whether pollination drops in fossil gymnosperms functioned as nectar is not clear, although the Lyginopteridales show some early evidence for insect interactions based on the presence of glands on both vegetative and reproductive structures [Oliver, 1909; reviewed by Labandeira et al. (2007)], which today often function in plant-animal interactions. There is better support for this later in the fossil record, e.g., Medullosalean prepollen grains were too large for wind pollination (Schwendemann et al., 2007). A consensus for Mesozoic insect pollination has been growing with mounting evidence based on new insect and plant fossils (Ren et al., 2009; Labandeira, 2010). According to Labandeira et al. (2007), early pinopsids such as Cheirolepidiaceae have structural modifications that are suggestive of insect pollination, implying that insects were attracted by pollination drops. Since nectar formation in modern gymnosperms is not associated with obvious nectaries, but is a nucellar product, the anatomical fingerprint is the nucellus. The basis for believing that nectar was possible in the past is based on the range of sugar concentrations that can be produced by the modern gymnosperm nucellus. Sugar concentrations in insect-pollinated modern gymnosperms are similar to those of insect-pollinated angiosperms; even wind-pollinated conifers produce, depending on species, a broad range of carbohydrate concentrations (Nepi et al., 2017). There is no reason to assume that such flexibility in carbohydrate production

by ovule nucellus could not have existed in the past. Additional support for the presence of a PCM α style drop comes from wind pollination experiments performed on models of several early seed plants (Niklas, 1983). Several species were shown to have relatively inefficient wind-based capture based on their morphology. Given caveats, it must be plausible to consider that some of these earliest seed-plants could have had animal-assisted pollination.

DROP DYNAMICS

A number of physiological characteristics of pollination drop behavior contribute to wind and insect pollination syndromes in modern gymnosperms. Some of these elements are of importance in imagining how the early gymnosperms described in the previous section reproduced. Understanding drop dynamics is also important if we are eventually to understand nectar dynamics.

On the surface of it, drop behavior appears to be simple: a pollination drop is secreted, and sooner or later, it retracts (**Figure 6**). Drops form prior to pollination and retract when they are pollinated by wind or insect, with the exception of some Pinaceae (Owens et al., 1998). Most of the information that we have on behavior is based on pollination drops that are readily accessible and easily viewed, such as those of PCM α species. Species in which ovules are deep within strobili and hidden from view are more difficult to study, e.g., Pinaceae, Taxodiaceae and Sciadopityaceae (Tomlinson, 2012). In this section, we will consider plant behavior in terms of the pollination drop functions of pollen capture and germination. Some examples of species for which we have nectar-specific information with respect to capture and germination will be discussed also.

Pollination Drop Secretion and Retraction

Pollination drop formation in various species either occurs prior to pollination (PCM α, non-phyllocladoid γ) or after (PCM β). Pollination drops originate in the nucellus, as shown by immunolocalization studies of pollination drop proteins (Poulis et al., 2005).

Regulation of Secretion

The secretion period may vary according to pattern and length. Secretion is often considered to be diurnal in nature (McWilliam, 1959; Strasburger, 1871), but as more phenological studies are carried out, an appreciation of the complexity of secretion has developed. Some species secrete their drops only during the day, e.g., *Cephalotaxus* spp., podocarpaceous conifers (Tomlinson et al., 1991), *Z. furfuracea* (Tang, 1987). Other species produce drops at night, such as those of nocturnally pollinated species of *Ephedra* (Rydin and Bolinder, 2015), and *Gnetum* (Kato et al., 1995). Unpollinated drops may last many days before retracting, e.g., 5 days in the case of *Taxus chinensis* (Xing et al., 2000) and up to 12 days in *Juniperus* (Mugnaini et al., 2007b). According to Dörken and Jagel (2014), pollination drops of cupressaceous conifers are present both day and night. There is no evidence of diurnal rhythms in secretion and retraction for the over twenty species that they investigated. In contrast to this apparent absence of a diurnal pattern is the example of a cupressaceous conifer with a far more complex pattern in which diurnal secretion is only one part of a longer pattern that spans days. In *Chamaecyparis nootkatensis,* drops are secreted during the night and then retracted the next day. This pattern is repeated for the first few days of the pollination period, but then a drop is secreted that lasts for many days and nights without retracting, before its final retraction ends the pollination period

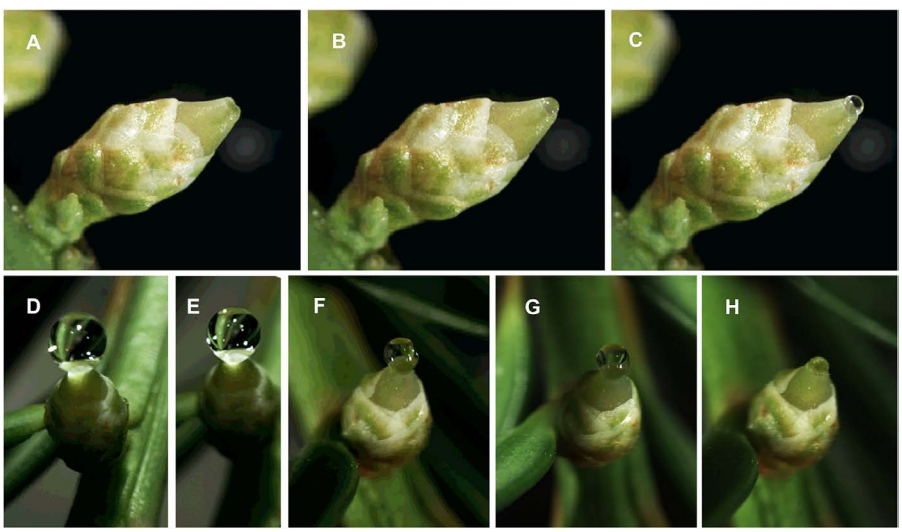

FIGURE 6 | Photographs from time-lapse study of *Taxus* x *media* pollination drop activity; photos by S. Gagnon. The series show pollination drop reformation after removal of initial drop: **A** time 0; **B** 50 min; **C** 100 min. The series show pollination drop retraction: **D** time 0; **E** 1 h, when pollen was dusted onto the drop and ovule using a syringe; **F** – 5 h; **G** – 20 h; **H** – 23 h.

(Owens et al., 1980). Cones of *L.* x *marschlinsii* produced post-pollination prefertilization drops in rhythms that were independent of the diurnal water potential patterns of the trees to which they were attached, which led to the conclusion that at least in some species the regulation of secretion is controlled at the cone and even the ovule level (O'Leary and von Aderkas, 2006).

These basic secretion patterns also occur together with other aspects of pollination syndromes. Insects are attracted by rewards and by smell. In *G. gnemon,* which has an ambophilous pollination syndrome, nocturnal moths are attracted by putrid volatiles released from strobili. *G. cuspidatum* attracts nocturnal flies with smells that recall rotten wood and fungi (Kato et al., 1995). *G. gnemon* secretes its pollination drops in the early evening in concert with the release of volatiles. The drops are retracted in the early morning. This pattern repeats itself. In insect-pollinated *Macrozamia* species, female strobili release volatiles at specific times of day that are coordinated with thermogenesis as part of the complex 'push-pull thermogenesis' system that controls pollination (Terry et al., 2007).

Regulation of Retraction

Retraction of pollination drops is governed by internal ovule physiology, external factors such as atmospheric evaporative demand and presence of pollen. Ziegler (1959), who studied *Ephedra* and *Taxus* concluded that retraction was strictly regulated by evaporation. In contrast, Tomlinson (2012) noted that the interaction between pollen and pollination drop was a more hydrodynamic process, triggering other processes that influenced water availability. Pollen enters the ovule, the micropyle of which rapidly seals shut, preventing further contamination by foreign pollen or microorganisms. Both pollen capture and ovule defense operate in concert with one another (Tomlinson, 2012). The entire ovule appears to be involved with retraction of pollination drops, as absorption experiments have shown using either Acid Fuchsin (Tison, 1911) or colchicine (Favre-Duchartre, 1958).

A study involving *Juniperus oxycedrus* (Mugnaini et al., 2007a) provides different evidence for retraction as a two-step process. As in the previous examples of cupressaceous given above, pollen of *J. oxycedrus* hydrates, loses its exine, triggering drop retraction. However, Mugnaini et al. (2007a) also found that foreign particles (beads, dust, foreign pollen [i.e., non-cupressaceous pollen]), as well as non-viable homospecific pollen caused an initial small diminution of the pollination drop, which was only followed by complete retraction if the pollen was of a cupressaceous species. This is an interesting result, as it should be recalled that unpollinated *Juniperus* pollination drops remain unretracted for up to 12 days, but once pollinated, retract in just minutes. In essence, this prevents entry of foreign objects into the ovule, which again points to pollination drops playing a role in ovule defense.

Pollen may also affect retraction in other ways. Xing et al. (1999) removed pollination drops from one cupressaceous species only to replace them with pollination drops from another cupressaceous species. The "replacement drops" receded when pollinated, but took much longer for complete withdrawal. However, the rate of retraction could be increased in proportion to the number of pollen grains added. The authors stated that pollination drop withdrawal is due to pollen regulation of the secretion process. This points to an effective recognition system for pollen by the ovule, possibly mediated via the nucellus. Further support for a recognition system comes from a comparison of retraction rates of pollination drops dusted with pollen sourced from evolutionarily close species to retraction rates of pollination drops dusted with pollen from distant species (Dörken and Jagel, 2014). The closer the evolutionary distance of the pollen, the faster the retraction response of cupressaceous pollination drops. However, it is not clear what the advantages of speed are. These sporophyte-gametophyte interactions, i.e., between nucellus and pollen, appear to carry a cost. The advantage of such a rapid retraction belies the lack of discrimination. Once pollinated, cupressaceous conifers do not initiate a new secretion, which means that capturing closely related but "wrong" pollen results in inevitable reproductive failure.

Pollination Drop Replacement

An important question in pollination drop physiology is drop replacement. Rain, sudden movement, and high evaporative demand can cause drops to disappear or be removed. In the case of nectar, non-pollinating insects can remove drops. In all of these cases, gymnosperm reproduction would be brought to a standstill if drops could not be replaced. If the one-and-only drop fails to collect pollen, then no other drop is produced and reproduction would fail. However, many species have drop replacement. *Thujopsis dolobrata* pollination drops can be replaced a maximum of eight times in succession (Dörken and Jagel, 2014). In short, the loss of a given drop does not lead to loss of function of the ovule, as it is able to replace the drop. In insect-pollinated species, replacement of drops is an important consideration, as the secretion that follows removal by an insect must play a role in scavenging pollen left at the rim of the micropyle by the pollinator. *E. aphylla* continues to produce pollination drops after pollen has already been captured from insects by an earlier drop (Moussel, 1980).

Pollination Drop Volume

Another aspect of pollination drops that has a bearing on pollination syndromes is drop volumes. Micropyle volume varies in species that have been measured, e.g., *P. menziesii* (Takaso and Owens, 1996b; von Aderkas and Leary, 1999a) and *L. x marschlinsii* (von Aderkas and Leary, 1999b). Of greater biological importance is the fact that pollination drop volumes vary between species. Insect-pollinated species in which pollination drops are functioning as nectar have much larger drops than insect-pollinated species in which only pollen is the reward. For example, pollination drops of *Gnetum,* a group that uses nectar as its primary reward, are in the 150–200 nL range (Kato et al., 1995), whereas pollination drops of cycads, a group that uses pollen as its primary reward, have volumes an order of magnitude less (Prior, 2014). Pollination drops of wind-pollinated species have small volumes (20–100 nL). There are some exceptions, such as *Taxus* spp., which have drops around

250 nL in volume (Nepi et al., 2009). There is also another type of exception, one that is particular to cupressaceous conifers. *Fitroya cupressoides*, *Cupressus sempervirens* (Dörken and Jagel, 2014), and *Chamaecyparis nootkatensis* (Owens et al., 1980) have cones in which the ovules are arranged so close to one another that synchronously secreted pollination drops fuse to form large amorphous drops. It has not been tested whether these 'super-drops' provide any advantages in pollen delivery efficiency or reproductive success.

Nectar viscosity may have an additional influence on insects. In *Gnetum*, nectar produced by sterile ovules on male strobili has a relatively low viscosity (Nepi et al., 2017) and tends to run and seep onto other structures, such as collars (Rydin et al., 2010). Insects are attracted to both the pollination drops and the run-off of these drops (Kato et al., 1995; Rydin et al., 2010). It would be worth testing whether the additional location of the nectar attracts nectar-seeking pollinators for a longer period, thereby contributing to greater reproductive success. Modeling micropyle and pollen chamber volumes may also be important for inferences of fossil plant biology.

Speed of Retraction

The speed of retraction varies. In *Taxus,* retraction following pollination takes 24 h (**Figures 6D–H**). Such a slow drop retraction may be entirely caused by evaporation (Xing et al., 2000). *Ginkgo biloba* is faster, taking only 4 h (Jin et al., 2012b). This slightly speedier process in *Ginkgo* is not solely caused by evaporation, but may also involve some undisclosed active process (Jin et al., 2012b). Active processes are thought to occur in two steps, the first of which involves pollen hydration and loss of its exine. The next step–active retraction–occurs as the pollen sinks into the drop (Lu et al., 2016). One family of conifers–Cupressaceae– is noteworthy in the rapidity with which pollination drop retraction takes place following pollination. Previous researchers had noted that species such as *Cephalotaxus drupacea* (Chesnoy, 1993), *Platycladus orientalis*, *Thuja occidentalis* (Xing et al., 1999), and *Chamaecyparis nootkatensis* (Owens et al., 1980) took less than 20 min. A broad survey in which pollination drop retraction times were measured in a few dozen cupressaceous species in response to pollen of another cupressaceous species, *Thujopsis dolobrata,* showed that retraction occurred, on average, in less than 10 min (Dörken and Jagel, 2014).

Nectar Retraction

In some species, pollination drops that act as nectar retract in response to pollination. *G. biloba* (PCM α), a putatively insect-pollinated species (Nepi et al., 2017), retracts its drop with a definite finality following pollen capture (Xing et al., 2000), but, as mentioned previously, other species with PCM α, such as *E. aphylla,* are able to produce pollination drops repeatedly following successful pollination, as well as after removal of the drops by insects (Moussel, 1980).

Pollen Germination

Pollination drops induce germination of pollen *in situ*, e.g., *Ephedra* (Moussel, 1980), *Pinus* (McWilliam, 1959) and *in vitro*, e.g., *Ephedra* (Mehra, 1938; Moussel, 1980) and *Taxus baccata* (Anhaeusser, 1953). Pollination drops deliver pollen to the nucellus, where it germinates, e.g., *L. decidua* (Villar et al., 1984), *Cephalotaxus drupacea* (Chesnoy, 1993). The nucellus is a complex tissue from a secretion standpoint. In a developmental study of the nucellus of *C. drupacea* over the course of pollination drop secretion, it was noted that at the beginning glyco-proteins and polysaccharide substances were released from the nucellar apex, and that at the end proteins and lipids were secreted (Seridi-Benkaddour and Chesnoy, 1991). The early substances, those secreted during pollination, influence pollen development. In studies of intergeneric crosses, pollination drops induce germination of homospecific pollen, whereas heterospecific pollen germination is less successful, as was shown in a study of intergeneric crosses of *Larix* and *Pseudotsuga* (von Aderkas et al., 2012). That is not always the case, as pollen of any given *Pinus* spp. will readily germinate in the ovule of any other species: selection becomes obvious only as tubes begin to grow inside the nucellus (McWilliam, 1959). Homospecific pollen tubes grow normally and fertilize the eggs, whereas heterospecific pollen tubes lose their way. Such selective abilities for nucellus and its liquid secretion points to the fact that in some gymnosperms pollination drops are capable of recognition at a species level. There is some evidence to support the idea that some gymnosperms have either preadaptation or adaptations for mate selection of pollen (Willson and Burley, 1983). Recently, transcriptomic study of *C. sinensis* ovules during pollination drop secretion revealed a transcript that matched an S-locus lectin protein kinase, as well as four transcripts that matched a g-type lectin S-receptor-like serine /threonine kinase (Pirone-Davies et al., 2016). More work needs to be done on the molecular interactions of S-receptor-kinases in gymnosperms, if only because in some flowering plants, sporophytic self-incompatibility systems in Brassicaceae make much use of S-receptor kinase. They function as female determinants of male rejection.

PERSPECTIVES

The purpose of this review was to summarize the many facets of sexual fluids in gymnosperms. Some aspects of these fluids are much better understood than others. To help future researchers in this area, we provide a number of points that we think are worth pursuing, if only to help shed further light on some of these highly successful adaptations of seed-plant reproduction.

Nucellus Is the Major Filter of Reproduction in Gymnosperms

What are the essential molecular events within the nucellus with regards to pollination drop secretion? Gene expression studies, and proteomic profiles – the useful first steps to developing models of nucellus activity and regulation of prezygotic reproductive events – have yet to be undertaken. The nucellus is a workhorse of a tissue that not only is responsible for megasporogenesis, pollination drop secretion, pollen tube screening, but also part of megaspore wall formation and, later, ovular plug formation. Compared to the integument,

which plays a much less active role, the nucellus is responsible for the bulk of ovule defenses and pollen-ovule interactions. How do nectar secretions in gymnosperms, i.e., pollination drop production, compare with the types of secretion by angiosperms? Can secretions be categorized according to known types, i.e., eccrine, merocrine (Vassilyev, 2010; Roy et al., 2017)? Do the processes that produce non-nectar differ from those that produce nectar? How extensively does the nucellus make use of enzymes that are widespread in angiosperm nectar regulation, such as invertases, e.g., CWIN4 (Heil, 2011), and sugar-transporters, such as SWEET9 (Roy et al., 2017)? Applying proteomics methods to pollination drops (Prior et al., 2013) has yielded many clues about pollination drop function. Such surveys should be expanded to include important clades, such as *Ginkgo*, cycads, and Podocarpaceae. The nucellus is not just involved in secretion, but also in resorption. How is resorption of pollination drops regulated? It seems that there are a number of possibilities, including slow responses, e.g., evaporation, and rapid responses. Do the latter involve ligand-gated ion channels?

Molecular Clues in Nectar-Based Pollination Drops

In part because gymnosperm secretions have historically been considered to be abiotic or involved in gametophyte interaction only, it becomes important now to consider what other compounds are found in pollination drops, especially those drops that function as nectar. Analysis of lipids, terpenoids, and phenolics, all of which are known to occur in angiosperm nectar, have yet to be carried out on gymnosperm nectar. Measurements of phosphates (Ziegler, 1959) and volatile organic compounds that attract insects and geckos (Kato et al., 1995; Celedón-Neghme et al., 2016) need to be done. Analysis of compounds involved in animal pollination, which we now know extends back to mosses and ferns (Cronberg, 2012), should be initiated.

Evolution of Time-Span Between Pollination and Fertilization

Compared to angiosperms, most gymnosperms invest more heavily in their prefertilization ovules. This adds developmental time (Leslie and Boyce, 2012) as a component of consideration compared to angiosperms in which the longest time from pollination to fertilization (i.e., vanilla orchid) is comparable to that of the fastest gymnosperms like *Ephedra* (Williams, 2012). As a result, the period between pollen capture and fertilization in a typical gymnosperm is relatively long. In more than a dozen genera it takes a year or more from pollen capture to gamete delivery (Willson and Burley, 1983; Williams, 2012). So how is it that some Gnetales with a PCM α-type pollination drop can trigger germination within a day of pollen capture (El-Ghazaly et al., 1998)? What are, in fact, the molecular controls of germination? What are the advantages and disadvantages of these various pollination to fertilization periods?

Ancient Origins of Gymnosperm Sexual Fluids and Nectar

From what we now know about ovule evolution, we can pose some new questions. Did the earliest Paleozoic seed plants such as hydraspermans have one or two sexual fluids? Did the earliest plants in the Devonian release sperm immediately upon capture of their prepollen, or was prepollen held for a time before release of swimming sperm? Was the fertilization fluid associated with a reproductive system in which microgametophytes reached maturity long after pollination before fertilizing eggs in later developed megagametophytes, as is seen in modern cycads and *Ginkgo*, or was the fertilization fluid part of single multi-purpose fluid in which the sexual fluid would have functioned as a PCM and as a fertilization fluid? If it was the latter, then it would suggest that the earliest ovules produced a single fluid having the functions of prepollen capture, delivery, germination, and ovule defense, as well as the function of a swimming sperm medium.

CONCLUSION

The two general types of sexual fluids in gymnosperms are pollination drops and fertilization fluids during fertilization. Both occur in ovules. The fertilization fluid originates from gametophytic tissues. We know less about these particular fluids in modern seed-plants, because we still await chemical analysis of their composition. We know much more about pollination drops. The plesiomorphic pollination syndrome of modern gymnosperms may share features with those of the earliest gymnosperms (i.e., PCM α). Pollination drops represent a significant investment in a fluid by the sporophytic tissues of the ovule. Drops have numerous functions in relatively complex PCMs: they ensure pollen capture, transport, germination and selection, ovule defense, and in some species, nectar reward for pollinators. The ability to present the drop as a nectar is found in three of the four major extant clades of gymnosperms, including the two most ancient ones (Ginkgoales, Cycadales). Nectar production may well have also been present in the distant past. We are beginning to understand elements of drop physiology, such as secretion and retraction. As we increase our knowledge of the regulation of secretion we will also begin to broaden our appreciation of nectar secretion by ovules as a unique and important contribution of gymnosperms to the evolution of seed plants.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Nectar in Plant–Insect Mutualistic Relationships: From Food Reward to Partner Manipulation

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It has been known for centuries that floral and extra-floral nectar secreted by plants attracts and rewards animals. Extra-floral nectar is involved in so-called indirect defense by attracting animals (generally ants) that prey on herbivores, or by discouraging herbivores from feeding on the plant. Floral nectar is presented inside the flower close to the reproductive organs and rewards animals that perform pollination while visiting the flower. In both cases nectar is a source of carbon and nitrogen compounds that feed animals, the most abundant solutes being sugars and amino acids. Plant–animal relationships involving the two types of nectar have therefore been used for a long time as text-book examples of symmetric mutualism: services provided by animals to plants in exchange for food provided by plants to animals. Cheating (or deception or exploitation), namely obtaining the reward/service without returning any counterpart, is however, well-known in mutualistic relationships, since the interacting partners have conflicting interests and selection may favor cheating strategies. A more subtle way of exploiting mutualism was recently highlighted. It implies the evolution of strategies to maximize the benefits obtained by one partner while still providing the reward/service to the other partner. Several substances other than sugars and amino acids have been found in nectar and some affect the foraging behavior of insects and potentially increase the benefits to the plant. Such substances can be considered plant cues to exploit mutualism. Recent evidence motivated some authors to use the term "manipulation" of animals by plants in nectar-mediated mutualistic relationships. This review highlights the recent background of the "manipulation" hypothesis, discussing it in the framework of new ecological and evolutionary scenarios in plant–animal interactions, as a stimulus for future research.

Keywords: nectar, partner manipulation, secondary compounds, mutualistic relationships, exploitation, plant–animal interactions

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INTRODUCTION

Mutualistic inter-species relationships, i.e., relationships in which interacting species reciprocate benefits received, are very common in all kingdoms of living organisms since virtually every species is involved in one or more such relationships. Mutualism has a pivotal role in the functioning of all current ecosystems and in key events of the evolutionary history of life on our planet, such as

the evolution of eukaryotic cells, colonization of land by plants and the radiation of angiosperms (Bronstein et al., 2006; Douglas, 2010; Bronstein, 2015).

Mutualisms have often been reported as evidence of the ancient classical theory of "balance of nature" that is rooted in Greek philosophy and mythology. According to this theory natural systems tend to remain in a stable equilibrium where natural forces prevent species from becoming too abundant or becoming extinct (Egerton, 1973). Mutualism, regarded as reciprocal cooperation between species, was therefore perfectly framed in this theory. The balance of nature was challenged by the evolutionary theory based on natural selection elaborated by Darwin (1859), according to which "natural selection cannot possibly produce any modification in a species exclusively for the good of another species." From a more recent evolutionary point of view, mutualistic relationships hide an apparent paradox since each species tends to maximize its own fitness when interacting with another and unrelated partners may have conflicts of interests (Sachs, 2015). These conflicts challenge the maintenance of mutualisms and selection may favor exploitation or the abandonment of such relationships. However, possible conflicts can be managed and mutualism stabilized in different ways, from special rewards for cooperatives and sanctions for cheaters to strict specificity in partner choice (Douglas, 2008, 2010). An additional possibility is to rely on some form of coercion/manipulation of the partner without disrupting the mutually beneficial outcomes of the relationship (Grasso et al., 2015; Heil, 2015a). From this point of view, mutualisms can best be regarded as reciprocally exploitative interactions that provide a net benefit to both parties. The net effect to each partner is highest when the benefit is maximized in relation to investment (Bronstein, 2001 and references therein).

Plants are involved in a myriad of mutualistic interactions with very diverse organisms such as bacteria, fungi and animals. Mutualisms with bacteria (nitrogen-fixing bacteria) and fungi (mycorrhiza) increase nutrient uptake by plants as well as providing organic matter and a suitable ecological niche to the heterotrophic counterpart. Since plants are anchored to the ground and have limited possibility of movements, the benefits they receive in mutualistic interactions with animals, and especially insects, arise from insects' ability to cover long distances. The animal's ability to move is involved in two processes invaluable for plant survival: dispersal of propagules, mainly pollen and seeds, and indirect defense against herbivory (Schoonhoven et al., 2005; Rico-Gray and Oliveira, 2007).

Pollen, the male gametophyte of seed plants containing the male gametes, needs to be transported from the anther to the stigma of a compatible carpel, a process called pollen dispersal or pollination that is the first step toward fertilization in all seed plants. According to a recent global estimate, 87.5% of all angiosperms are pollinated by animals (Ollerton et al., 2011) and a significant fraction of gymnosperms are ambophilous, i.e., pollinated by wind and by animals as well (Nepi et al., 2017). Insects are the most numerous and diverse animals involved in pollination (Ollerton, 2017). Besides its importance from an ecological and evolutionary perspective, pollination has great economic value: more than one third of human food resources are derived from insect pollination and about 1500 crop species worldwide are pollinated by insects, so the estimated economic value of this ecosystem service adds up to $360 billion (Hanley et al., 2015).

Being sessile and having limited movements, plants have developed an array of defense strategies against predation by herbivores (Schoonhoven et al., 2005). Direct defenses involve morphological and chemical cues that discourage herbivores from feeding on a plant. Plants may also engage in mutualistic relationships with arthropods, such as ants, wasps, spiders, mites, and parasitoids, that patrol the plant and deter or even kill herbivores (Arimura et al., 2005; Dicke and Baldwin, 2010; Heil, 2015a). The plant defends itself indirectly by attracting an animal "body-guard" via a tritrophic interaction (Heil, 2008). Indirect defense based on mutualism with ants, on which we focus in this review, has wide phylogenetic and geographic distribution, although the highest level of complexity and coadaptation of plant–ant relationships is reached in angiosperms of tropical and subtropical regions (Hölldobler and Wilson, 1990; Heil and McKey, 2003; Rico-Gray and Oliveira, 2007; Ness et al., 2010). Indirect defense involving ants is very efficient and has also evolved outside the plant kingdom: aphids (Hemiptera, Aphididae) as well as caterpillars of certain species of blue butterflies (Lepidoptera: Lycaenidae) are protected indirectly by ants against their predators (Nepi, 2017 and references therein).

Irrespective of the type of mutualism, whether for pollination or indirect defense, the benefit earned by the animal is generally a food resource produced by the plant, in most cases nectar. Nectar involved in mutualistic relationships with pollinators is called floral nectar (FN, **Figure 1**) since it is produced by organs (nectaries), usually inside the flower close to the reproductive organs, whereas nectar involved in indirect defense is generally offered in the vegetative part of the plant and is known as extra-floral nectar (EFN, **Figure 1**). Most insect pollinated angiosperms produce FN as the main primary floral attractant and their floral nectaries vary widely in position, shape and structure (Galetto, 2007). EFN is reported in about 4000 plant species (with estimations up to 8000 plant species), which are distributed among 457 independent lineages and living in a wide variety of tropical, subtropical and temperate habitats (Marazzi et al., 2013; Weber and Keeler, 2013). Both types of nectar, being sugary water-based acellular secretions, are easily collected, ingested, digested and absorbed by an extraordinary variety of animals, making it a ready-to-use energy source (Nicolson, 2007). Thus for 100s of years nectar-based plant–pollinator relationships (and subsequently plant–ant mutualism) have been reported as examples of symmetric mutualism: services provided by animals to plants in exchange for food provided by plants to animals. These cooperative relationships fit into the "balance of nature" theory, a perspective that still permeates modern ecology textbooks and papers that frequently refer to nectar as a "reward" for pollinators or plant defenders, attributing an exclusively cooperative meaning to such interactions. However, mutualisms may also be established on a selfish basis, limited by costs and driven by conflicts of interest between partners (Bronstein et al., 2006). Conflicts of interest between interacting partners clearly characterize nectar-mediated plant–animal interactions:

FIGURE 1 | Arthropods feeding on floral nectar (FN) **(top)** and extra-floral nectar (EFN) **(bottom)**. *Bombylius* sp. probing for nectar in a flower of *Echium italicum* (top left; picture by Sara Mancini, Department of Environmental Sciences, University of Siena); *Eristalis tenax* foraging for nectar on the flowers of *Scabiosa* sp. (top right; picture by MN, Department of Environmental Sciences, University of Siena); *Crematogaster scutellaris* feeding on EFN produced by stipular nectaries of *Vicia sativa* (bottom left; picture by Daniele Giannetti and DG, Myrmecology Lab, University of Parma); *Temnothorax* sp. collecting nectar from a foliar nectary of *Pteridium aquilinum* (bottom right; picture by Daniele Giannetti and DG, Myrmecology Lab, University of Parma).

plants target efficient service (pollination or indirect defense) by nectarivores at the lowest possible cost (thus minimizing the quantity of nectar they produce), while animals are interested in obtaining good quality food in sufficient quantity (nectar) irrespective of whether pollination or indirect defense of the plant is involved. For example, animals can detect humidity gradients over flowers that enable them to assess the amount of FN without probing the flowers and touching the reproductive organs (von Arx et al., 2012). In this scenario, selection would tend to favor exploitation of mutualism (Sachs, 2015) and examples of pure exploiters are well-known on both sides. Although orchids are insect pollinated, about one third do not produce any kind of food (Ackerman, 1986). The flowers of these nectarless orchids rely on several types of mimicry to attract insects, including specific resemblance to flowers of nectar-producing species (Johnson, 2000; Jersáková et al., 2006). Insects are not able to discriminate the flowers of the two species and visit the flowers of the nectarless orchid by mistake (Johnson, 2000). On the other hand, nectar robbing by insects that do not perform pollination has been known since the early observations of bumblebees stealing nectar from flowers of *Pentstemon*, *Antirrhinum*, *Stachys,* and *Salvia* (Darwin, 1841). Pure exploiters are also known in plant–animal relationships involving indirect defense. For example, ants of the genus *Cataulacus* (*C. mckeyi*) exploit the EFN of *Leonardoxa africana* without protecting the plants from herbivores (Gaume and Mckey, 1999). Nonetheless, the costs and benefits for both partners associated with cheating are not always univocal and cheating may sometimes not have detrimental effects (Maloof and Inouye, 2000, see below). Beyond pure exploitation or cheating, relationships with mutually beneficial outcomes are even subject to selective pressure to maximize the benefits obtained by one partner while still providing the reward/service to the other partner. These strategies can be considered more nuanced styles of exploitation than pure cheating, since the mutualism has

costs for both partners. It was recently demonstrated that such interactions are (or may be) mediated by specific nectar compounds (Wright et al., 2013; Heil et al., 2014; Nepi, 2014; Grasso et al., 2015; Baracchi et al., 2017). Since most of these compounds modify insect physiology and behavior, this recent evidence has motivated researchers to regard them as a form of "manipulation" of animals by plants, namely mutualisms with a coercive component.

The aim of this review is to disentangle the complex ecological and evolutionary scenario recently revealed in nectar-mediated plant–animal interactions by considering mutualism, cheating and exploitation in a wider ecological framework and by analyzing the background of the "manipulation" hypothesis. Manipulative strategies seem to be more common in mutualistic relationships than was previously thought and they can be regarded as adaptations to counteract the temptation to cheat with the ultimate effect of stabilizing the mutualism (Heil, 2015b).

NECTAR PRODUCTION IS COSTLY FOR PLANTS

In the evolutionary history of plants, nectar first appeared in pteridophytes (Schuettpelz and Pryer, 2008). Nectaries of pteridophytes are located on the fronds and are known as foliar nectaries (Koptur et al., 2013). They can therefore be considered topographically analogous to the EFNs of angiosperms. According to the exploitation hypothesis (*sensu* Del-Claro et al., 2016) derived from early physiological studies (Nieuwenhuis von Üxküll-Güldenband, 1907; Zimmermann, 1932), nectar was secreted as a "waste product" of excess carbohydrates, a theory that was probably inspired by Darwin (1859), since in On the origin of species, he wrote "certain plants excrete sweet juice, apparently for the sake of eliminating something injurious from the sap." This hypothesis was recently re-considered by De la Barrera and Nobel (2004). Speculating on the origin of nectar secretion, these authors proposed two alternative hypotheses. The "sugar excretion" hypothesis proposes that nectar production arose to remove excess solutes supplied by the phloem and is triggered by intense transpiration of developing organs, somehow similar to the original physiological hypothesis. The "leaky phloem" hypothesis argues that nectar secretion is a leakage of phloem solution, resulting from the structural weakness of developing tissues exposed to high pressure in the phloem (De la Barrera and Nobel, 2004). Both hypotheses are in contrast with the different composition of EFN (and even FN) and phloem sap found in some extant species (Keeler, 1977; Nicolson and Thornburg, 2007) but they could be in line with the early appearance of nectar in pteridophytes. Today foliar nectaries are found in two clades of pteridophytes (Marattiales and leptosporangiate ferns) dating to the Palaeozoic (Schuettpelz and Pryer, 2008). Since ants originated in the early Cretaceous, 135–115 Mya (Ward, 2007), EFN probably initially had a function not involving ants. The latter began to exploit the sugary secretion soon after their origin (Nepi et al., 2009). Foliar nectar of extant ferns can be considered functionally similar to the EFN of angiosperms, since it may be involved in recruiting ants that protect against herbivores, although this function is more variable and controversial than in angiosperms (Koptur et al., 2013). Angiosperms, which evolved and radiated in the early-middle Cretaceous, reinforced nectar-mediated interactions with animals by adjusting the chemical, physiological and phenological traits of nectar in relation to the needs of new co-evolving insect groups (Nepi et al., 2017). These new adaptations presumably imply a higher cost of nectar production than for the "leaky phloem" or "sugar excretion" hypotheses of "early" nectar. Estimates of FN production costs in extant species in terms of daily photosynthate vary from 3.3% in short-lived flowers to 37% in long-lived flowers (Southwick, 1984). A trade-off between nectar production and plant growth-reproduction has also been demonstrated (Pyke, 1991). Although such estimates are not available for EFN, a cost for its production can be assumed, since there is evidence that FN and EFN are produced by the same general mechanisms (Heil, 2015a) and the composition of both types may differ from that of phloem sap (Keeler, 1977; Nicolson and Thornburg, 2007 and references therein). Indirect evidence of the cost of EFN and FN is that it is often reabsorbed if not consumed, and the carbohydrates are presumably allocated for other purposes (Búrquez and Corbet, 1991; Nepi and Stpiczyńska, 2008; Escalante-Pérez et al., 2012).

UNCERTAINTY OF BENEFITS IN NECTAR-MEDIATED INTERACTIONS

The classical view of plant–animal interactions considers the services of pollination and indirect defense to be the main benefits for plants producing FN and EFN, respectively, whereas the animal counterpart obtains nutritious food. Nectar-producing plants have a higher probability of attracting insects that accomplish pollination and thus a higher probability of producing seeds. For example in several species exhibiting variability in nectar production between individuals, it was revealed that high nectar availability favors pollinator attraction, promoting floral visits and reproductive output, whereas decreased seed set was found in low nectar-producing individuals (Real and Rathcke, 1991; Galetto and Bernardello, 2004; Brandenburg et al., 2012; Cruz-Neto et al., 2015 and references therein).

In EFN-bearing plants, various ant-exclusion experiments clearly demonstrated (in most cases but not in all, see Sanz-Veiga et al., 2017 and references therein) an indirect function of EFN in protecting against herbivores and thus increasing plant fitness (reviewed in Heil, 2015a). The direct link between EFN, ants and plant defense is highlighted by induction of extra-floral nectary activity: herbivore damage may increase EFN production, increasing ant recruitment and raising protection (Heil, 2015a; Del-Claro et al., 2016 and reference therein). Interestingly, the activity of herbivores may affect interactions with pollinators (Adler et al., 2006; Rusman et al., 2018), so that ants defending plants against herbivores indirectly also protect plant interactions with pollinators, further improving plant fitness.

On the animal side, relatively few studies have assessed the benefits of feeding on nectar. Beyond being an energy source for insect flight by virtue of its high sugar content, specific nectar components are recognized as beneficial for pollinating insects. For example, amino acid-rich nectar improves butterfly fecundity (Mevi-Schütz and Erhardt, 2005). Proline, one of the more common and abundant amino acids in FN (Baker and Baker, 1983a), is required by honey bees for egg lying and increases the size of their hypopharyngeal gland acini (Darvishzadeh et al., 2015), organs that produce royal jelly. Oxidative degradation of proline is also used by some bees and wasps to fuel their flight (Carter et al., 2006; Teulier et al., 2016). Certain secondary metabolites detected in FN (such as gelsemine, anabasine, and nicotine) may benefit pollinators by increasing their resistance to parasites and pathogens (Roy et al., 2017; Stevenson et al., 2017 and references therein). EFN is reported to be a valuable resource for certain ant species, since it increases individual and colony growth rate and survival (Byk and Del-Claro, 2011). Increased survival and growth rate have also been reported for non-ant consumers, such as spiders and parasitoids, which additionally showed increased egg production and increased parasitization rate, respectively, after feeding on EFN (Heil, 2015b and reference therein).

In this general framework, the outcome of these interactions is highly conditional, varying in space and time and according to the species involved, partner behavior, environmental constraints and ecological context (Menzel et al., 2014; Hoeksma and Bruna, 2015; Del-Claro et al., 2016). The plants involved in nectar-mediated interactions with animals therefore pay the cost of nectar production for benefits that may not accrue.

For example, bumblebees (common pollinators of cultivated and native plants) sometimes rob nectar from flowers with long tubular corollas or spurs where the nectar is inaccessible (Inouye, 1983; Irwin et al., 2010). Robbing is a foraging strategy by which insects obtain nectar without contacting the reproductive organs of the flower and performing pollination. It is done by biting the base of a flower close to the nectar reservoir (primary robbing) or by exploiting perforations made by other animals (secondary robbing). Surprisingly, bumblebees rob FN in species that they could pollinate legitimately. This particular behavior could be due to obstacles to reaching nectar in the conventional way: hairs and structural barriers that hamper nectar access can often be avoided by unconventional routes to the nectar. Alternatively, large sticky pollen grains, which adhere to the body of insects visiting flowers in the conventional way can be bothersome and therefore promote nectar theft. It has been demonstrated that bumblebees finding robbed flowers significantly increased their behavior as primary robbers although they previously behaved as legitimate pollinators (Leadbeater and Chittka, 2008). Interaction with other bumblebees that practice secondary robbing can turn a legitimate forager bumblebee into a secondary robber. Since other insect species may also make holes to steal nectar, it seems likely that such interactions may involve heterospecific individuals (Leadbeater and Chittka, 2008 and references therein). Thus it appears that nectar robbing behavior may spread by social transmission through a community of insects, with plausibly negative effects on the plant community. However, robbers are not always detrimental, as frequently assumed for cheating since this term has a negative significance for humans. The frequency of negative, neutral and positive effects was actually equal in 18 studies that measured the effect of robbing on seed set (Maloof and Inouye, 2000) and the same robber species can have different effects on the reproductive success of distinct plant species (Bergamo and Sazima, 2018). Cheaters and robbers such as bumble bees and carpenter bees are also in some cases reported to be pollinators of the flowers they rob (Sampson et al., 2004; Zhu et al., 2010; Singh et al., 2014).

Insect behavior and ecological context are also responsible for indirect costs that may arise from ant-plant mutualism mediated by EFN. As nectar sources could be vital for individual nutrition and colony survival, some ants may also forage FN (Santos et al., 2014) but are generally not considered good pollinators because their metapleural glands produce anti-bacterial and anti-fungal secretions that disrupt the normal function of pollen grains (Peakall et al., 1990). They may have a negative effect on plant–pollinator mutualism by decreasing the quantity of FN available. Ants may also feed on pollen, reducing flower fertilization (Del-Claro et al., 2016). Furthermore, flower-visiting ants may deter and/or prey on pollinators, although this does not seem to affect the plant's fruiting (Assunção et al., 2014). In other cases, ant behavior may have a direct and extremely detrimental effect on plant reproduction. The ants *Allomerus cf. demerarae* and *Crematogaster nigriceps* "castrate" their host plants, the former removing flowers from *Cordia nodosa* and the latter pruning axillary shoots bearing the inflorescences of *Acacia drepanolobium* (Young et al., 1997; Yu and Pierce, 1998). In this way they promote vegetative growth of the host plant, which thus produces more domatia and EFN, to the detriment of plant reproduction. Nonetheless, this behavior may have a positive effect on plant fitness in the long term, since young plants can be preferred by ants that strongly promotes their survival. Once older, plants can be colonized by other ant species that do not sterilize them allowing their reproduction (Palmer et al., 2010). The overall effect may be an increase in plant fitness.

INSECT FORAGING ACTIVITIES ARE AFFECTED BY PLANTS THROUGH NECTAR TRAITS

The few examples reported above show that nectar-foraging behavior of animals may be unpredictable and highly variable, exposing nectar producing plants to the risk of not receiving any real benefit as a counterpart for the expense of nectar production. Selection can therefore be expected to favor strategies to counteract this risk. Plants have several ways of affecting the behavior of nectar foragers and study of these effects led to the first hypotheses about nectar-based manipulation of insects by plants (Biernaskie and Cartar, 2004; Pyke, 2016). The studies were almost exclusively focused on relationships between FN and pollinators, but the manipulation hypothesis was recently extended to EFN and ants (Grasso et al., 2015).

Plants Affect Pollinator Foraging Behavior by Providing a Highly Variable Nectar Source

The reproductive success of plant species that rely on pollination by insects is determined by insect foraging activity. The behavior of foraging insects determines which flowers set seed and the pattern of pollen transfer (and thus male gametes) between plants, and ultimately plant population genetic structure (Goulson, 1999).

The behavior of foraging insects involves decisions when encountering a food resource according to variability of nectar traits (such as volume and concentration) and their spatial distribution (Goulson, 1999; Leiss and Klinkhamer, 2005; Cnaani et al., 2006; Dreisig, 2012). The abundance and spatial distribution of nectar available to a foraging insect at a given time is called the nectar standing crop (Galetto and Bernardello, 2005). Nectar standing crop varies widely between flowers of a plant (Keasar et al., 2008). This variability is the combined result of the nectar production rate of flowers and insect foraging activity.

Plants may be under selection to produce variable nectar resources so as to economize investment in nectar production while increasing the possibility of cross-pollination. At population level, the nectar standing crop generally has a patchy distribution: one or more highly productive plants are neighbors to others that produce less (Leiss and Klinkhamer, 2005). The same happens at the smaller scale of individuals of nectar producing species that may bear a certain number of nectarless flowers (Gilbert et al., 1991; Bailey et al., 2007). Empty flowers borne by nectar-producing individuals are an energy-saving strategy that enables the plant to save resources normally allocated to nectar production while maintaining its attraction for pollinators (Bell, 1986). Nectar standing crop variability is also revealed by the generally positive skewed distribution of nectar production by individuals, which means that there are few flowers producing a large quantity of nectar and many flowers producing a smaller amount (Gilbert et al., 1991 and references therein). Nectarless and nectar-poor flowers can be considered a case of "partial cheating" when compared to the "total cheating" of deceptive nectarless plant species, reported above.

Standing crop structure (i.e., the abundance of nectar offered and its spatial distribution) affects both the duration of visits and distance between successive visits, since pollinators move quickly to more distant patches, individuals or flowers when they encounter nectarless or nectar-poor specimens (Gilbert et al., 1991; Smithson and Gigord, 2003; Leiss and Klinkhamer, 2005; Bailey et al., 2007). Short visits and fast moves between flower patches reduces the probability of geitonogamy (self-pollination between flowers on the same plant) and the risk of inbreeding. Highly variable standing crops are therefore considered a strategy to increase the out-crossing rate and offspring fitness (Smithson and Gigord, 2003; Bailey et al., 2007; Keasar et al., 2008). Moreover, plants offering high rewards may have an emanating effect on neighbors offering small rewards (Leiss and Klinkhamer, 2005). In this way plants with low nectar production may benefit from pollinator services enhanced by the presence of high nectar producing neighbors, while saving on the cost of nectar production.

Plants may exert control over nectar standing crop by providing highly variable nectar production that in turn affects the foraging behavior of pollinators. This outcome supports the idea that plants may "manipulate" the foraging behavior of pollinators to optimize pollen flow between individuals. In this framework a manipulation hypothesis was first elaborated by Biernaskie and Cartar (2004) who reported a positive correlation between variability in nectar production rate and floral display (number of open flowers) in individual plants of nine angiosperm species. According to these authors, the increased attractiveness of a plant caused by an abundance of flowers is coupled with greater variability in nectar production rates of its flowers so as to obtain an optimal trade-off between number of visits and the length of the pollinator visitation sequence.

Nonetheless, nectar standing crop is affected by two orders of variability: variability in nectar production controlled by plants, on which further variability generated by the foraging activity of pollinators is superimposed (Goulson, 1999; Keasar et al., 2008). Pollinator-generated variation seems to have major effects on pollinator foraging, possibly overriding the effects of plant-generated variation. Pollinator-generated variability in nectar resources may thus reduce the selective benefit of plant-generated variability as a strategy to decrease geitonogamy (Keasar et al., 2008). It is also worth noting that environmental parameters (at macro- and micro-environment level) may influence nectar production, standing crop and insect activity (Pacini and Nepi, 2007), further decreasing the strength of the control exerted by plants.

It follows that plant control of pollinator behavior through modulation of variable nectar production is possible but seems quite weak. Plants may, however, use other tools to influence the feeding behavior of pollinators.

Plants Control Foraging Behavior of Pollinators by Nectar Chemistry

A nectar trait quite recently considered when studying the effect of nectar on insect feeding behavior is its chemical composition. Floral and EFN is largely composed of sugars, usually together with other primary metabolites, such as amino acids, lipids, and proteins (Nicolson and Thornburg, 2007). Secondary metabolites (alkaloids, terpenoids, and phenols) are reported more rarely than primary metabolites, but their presence is presumed to be quite common (Nicolson and Thornburg, 2007; Roy et al., 2017; Stevenson et al., 2017). Nectar secondary metabolites include volatile compounds that impart scent to both floral and EFN, enabling insects to locate it (Raguso, 2004; Röse et al., 2006).

Both primary and secondary metabolites can have effects on insect behavior.

Effects of Primary Metabolites on Pollinators

Sugars and amino acids are the most abundant primary metabolites and are an important source of energy and nitrogen, respectively (Roy et al., 2017 and reference therein). They are

therefore the main determinant of the food value of nectar, but they can also affect the attractiveness of nectar since they are responsible for its taste (Gardener and Gillman, 2002). Both sugars and amino acids affect insect feeding behavior through post-ingestive signaling, involved in associative learning and memory (Simcock et al., 2014, 2018), processes that are of particular importance in making choices during foraging. Associative learning is a mechanism that allows animals to identify cues associated with nutrients so that they can be located quickly when required (Simcock et al., 2014).

Sucrose is the most common and abundant nectar sugar (Baker and Baker, 1983b) and is preferred by honeybees to other naturally occurring sugars (Barker and Lehner, 1974). Its concentration is an important determinant for many foraging-related decisions (Scheiner et al., 2004). Interestingly, this disaccharide is recognized as the most phagostimulatory sugar for honeybees, and bees rewarded with sucrose are more likely to learn to associate an odor with a food source (Simcock et al., 2018).

All twenty amino acids commonly found in proteins have been identified in various plant nectars. Proline seems to be of special importance for insects. It not only contributes a taste preferred by insects (Alm et al., 1990; Bertazzini et al., 2010), but also stimulates the insect salt cell, a labellar chemosensory receptor, resulting in increased feeding behavior (Hansen et al., 1998). In an experiment using free-flying foragers, Hendriksma et al. (2014) demonstrated that honeybees preferred essential over non-essential nectar amino acids. Phenylalanine, one of the most abundant amino acids in nectar (Petanidou, 2007), has strong phagostimulatory activity, while glycine is a phagodeterrent, both at concentrations similar to that occurring naturally in nectar (Hendriksma et al., 2014). The same authors also demonstrated a trade-off between sucrose concentration and amino acid preferences: nectar with low sucrose concentration that is normally unattractive to bees can become attractive if it contains minute concentrations of the phagostimulant phenylalanine, whereas the phagodeterrence of glycine can be masked by high concentrations of sucrose (Hendriksma et al., 2014). It follows that plants can replace expensive carbohydrates in their nectar with minute concentrations of phagostimulating amino acids, or modulate pollinator visits by adding phagodeterrent amino acids.

The link between sucrose and amino acids in affecting feeding behavior was also revealed by experiments testing how nutritional state affected the taste of specific amino acids (isoleucine, proline, phenylalanine, and methionine) and associative learning of honeybees (Simcock et al., 2014). Results showed that bees pre-fed sucrose solution consumed less of solutions containing amino acids and were less likely to associate amino acid solutions with odors. Surprisingly, bees pre-fed solutions containing an amino acid were also less likely to associate odors with sucrose the next day. Bees consumed more food and were more likely to learn when rewarded with an amino acid solution if they were pre-fed isoleucine and proline (Simcock et al., 2014). The authors concluded that single amino acids at relatively high concentrations decrease feeding on sucrose solutions containing them, and they can act as appetite reinforcers during learning.

Effects of Secondary Metabolites on Pollinators

Plant secondary metabolites (SMs) can be defined as "compounds that do not occur universally but are restricted to specific plant taxa, or occur in certain plant taxa at much higher concentrations than in others, and have no (apparent) role in primary metabolism" (Schoonhoven et al., 2005). Plants produce a plethora of SMs with a variety of functions. They are mainly involved in defense against herbivores and other enemies such as fungi and bacteria but may also have other additional functions (Schoonhoven et al., 2005). Secondary metabolites, including tannins, phenols, alkaloids, and terpenes, have been found in FN in more than 21 angiosperm families (Adler, 2000). These compounds have been known since the 1970s and were initially considered to be toxic deterrents of nectar thieves while encouraging specialist pollinators (Baker and Baker, 1983a; Adler, 2000; Barlow et al., 2017; Stevenson et al., 2017). More recently, researchers have discovered that these compounds, and particularly alkaloids, may play an important role in managing visitor behavior.

Nicotine (a pyridine alkaloid) is a typical insect-repelling alkaloid and is found in the FN of *Nicotiana attenuata,* where it increases the number of flowers visited and reduces the volume of nectar consumed by hummingbirds and moth pollinators (Kessler and Baldwin, 2007). The unpleasant taste of nectar containing nicotine reduces nectar consumption and the length of flower visits, leading to a higher rate of outcrossing (Kessler et al., 2012). Shorter visits also reduce the risks associated with excessive visitation of individual flowers, such as increased reception of incompatible pollen or removal of compatible pollen grains from the stigma surface (Pyke, 1984). Plants with FN containing nicotine are able to minimize nectar volumes, while maximizing pollination efficiency, seed production and plant fitness. In this perspective the function of nectar is not to increase flower attractiveness but rather to optimize pollen flow between individuals by altering the feeding behavior of insects. This outcome clarifies the apparent contrast between the general deterrent effect of SMs and plants' need to efficiently attract insects as vectors of pollen.

Other nectar SMs may have phagostimulatory activity, although this function seems restricted to species adapted to feed on plants with a high content of SMs (Stevenson et al., 2017). Note that SM effects on insects are dose dependent (Manson et al., 2013) and their concentrations in nectar may also be highly variable in a single plant; however, it is generally recognized that SM levels in nectar are lower than in other plant tissues (Cook et al., 2013).

The feeding deterrent function of nectar SMs is due to the unpalatable taste of alkaloids, especially nicotine, that is perceived by insects as soon as their proboscis contacts the nectar. The mouth parts of insects have contact chemoreceptors with neurons responding to sugars, salts, acid, water and non-nutrient compounds (Stevenson et al., 2017 and references therein). As in the case of amino acids (see above), chemoreceptor response

TABLE 1 | Secondary compounds and their hypothesized or tested post-ingestive effects on neurobiological or physiological traits of insects.

Compound	FN	EFN	Tested insect	Behavioral/physiological effects	Reference
Caffeine	×		Honeybees (*Apis mellifera*)	Increased learning and memory at nectar-level concentrations	Wright et al., 2013
Caffeine and theophylline			Ants (*Myrmica sabuleti*)	Increased linear speed, memory, and conditioning ability. Decreased consumption of food and precision of reaction.	Cammaerts et al., 2014
Nicotine	×		Bumblebees (*Bombus terrestris audax)*	Increased learning and memory at nectar-level concentrations	Baracchi et al., 2017
Cocaine			Ants (*Myrmica sabuleti*)	Increased audacity. Decreased linear speed, precision of reaction, response to pheromones and consumption of food. Inhibited conditioning ability. Induced dependence	Cammaerts et al., 2014
Atropine			Ants (*Myrmica sabuleti*)	Decreased olfactory perception and precision of reaction	Cammaerts et al., 2014
Non-protein amino acids (GABA, β-alanine)	×			Effects on muscle activity, nervous system, and phagostimulation	Nepi, 2014; Felicioli et al., 2018
Chitinase (nectar protein)		×	Ants (*Crematogaster*)	Inhibition of gut invertase	Heil et al., 2014

to nectar SMs is modulated according to sucrose concentration: rejection of high concentrations of SMs can be attenuated by high carbohydrate content of nectar (Köhler et al., 2012).

Nectar SMs may have post-ingestive effects on other targets in the insect body, such as the brain, affecting their neurobiology (**Table 1**). It was recently reported that honeybees rewarded with solutions containing caffeine (a purine alkaloid) at concentrations similar to that occurring naturally in the FN of *Coffea* and *Citrus* species, remembered the learned floral scent better than honeybees rewarded with sucrose alone (Wright et al., 2013; **Table 1**). Caffeine, an adenosine-receptor antagonist, affected Kenyon cells' activity, potentiating the response of honeybee brain mushroom body neurons that are involved in olfactory learning and memory formation (Wright et al., 2013 and references therein). At higher concentrations, caffeinated solutions exerted a deterrent effect and bees were more likely to reject caffeinated solutions. Pollinators therefore drive selection for nectar that is not repellent but still has neurobiological activity. The "increased memory" effect of nectar-like concentrations of caffeine may be one reason for unexplained flower constancy, frequently observed in foraging honeybees (Goulson, 1999). From the plant perspective, pollinator constancy is clearly beneficial since it minimizes pollen wastage and unfruitful heterospecific pollination.

A similar behavioral effect was reported in bumblebees fed with solutions containing nicotine at concentrations within or above the natural range (**Table 1**). Bumblebees were only deterred by unnaturally high nicotine concentrations (50 ppm) and this deterrence disappeared or became attraction at lower nectar-like concentrations (1 and 2.5 ppm) (Baracchi et al., 2017). The same concentrations affected bumblebee flower preference through enhanced memory of floral traits. Increasing numbers of bumblebees remained faithful to flowers containing nicotine at any tested concentration, even if they become a suboptimal choice in terms of caloric value (Baracchi et al., 2017). Although the neurobiological mechanism was not studied, it is postulated that nicotine, being an agonist of nicotinic acetylcholine receptors, may act as a psychoactive drug, modulating cholinergic neuron activity in the insect brain and positively reinforcing the flower-reward association (Baracchi et al., 2017 and references therein).

In addition to alkaloids, other nectar SMs such as non-protein amino acids (NPAAs), i.e., amino acids that are not used by organisms to build proteins, are potentially involved in modulating insect behavior (**Table 1**). Those more common in nectar, i.e., γ-aminobutyric acid (GABA) and β-alanine, are important insect nervous system neuromodulators (Nepi, 2014 and references therein). They may affect insect behavior in several ways: by affecting insect nervous system physiology, regulating nectar intake through phagostimulation and promoting muscle function (Felicioli et al., 2018). Among the NPAAs found in nectar, GABA seems of particular interest since in invertebrates GABA-receptors are located peripherally in muscle tissue and neuromuscular junctions bathed in hemolymph (Bown et al., 2006) and may be sensitive to variations in GABA levels caused by insect feeding on GABA-rich nectar. However, no clear confirmation of this hypothesis has yet been found.

Do Plants Control the Behavior of Ants by Means of EFN?

In the case of EFN, there is evidence that variations in nectar productivity between plant species and at different times of day may influence the visitation patterns of ants and in some cases also their numbers, showing the important key role of these nectaries in ant-plant interaction systems (Blüthgen et al., 2000; Lange et al., 2013, 2017). However, nectar quality and certain ant behaviors may also have important consequences for the organization and distribution of ant foraging activities

(Blüthgen et al., 2000; Blüthgen and Stork, 2007; Anjos et al., 2017). Compared to FN, the effects of EFN chemistry on ants (and other predators visiting plants bearing EFNs) has certainly been neglected. It has been reported that the unbalanced C/N ratio of nectar may increase the ants'attraction for *N*-rich food, and hence the likelihood that they will attack herbivorous insects on the host plant, contributing to indirect defense of the plant (Ness et al., 2009). Thus it appears that indirect plant protection involving ants is elicited by plant-mediated dietary imbalances. Actually, the aggressiveness of tending ants increases with increasing EFN carbohydrate content (Grover et al., 2007; González-Teuber et al., 2012) but there may be another explanation. Carbohydrates are a major fuel for metabolically expensive behaviors, such as ant aggressiveness and hyperactivity. In any case, higher and lower C/N ratios have been reported in response to herbivore activity, with EFN sucrose (Ness, 2003) and amino acid (Smith et al., 1990) contents both increasing after herbivore attacks. It has also been suggested that changes in the C/N ratio of EFN could manipulate the prey preferences of foraging ants: increasing EFN carbohydrate levels resulted in reduced feeding on high lipid prey (Wilder and Eubanks, 2010).

There is little literature on secondary metabolites in EFN. Trace amounts of the alkaloid harmine were reported in EFN of *Passiflora edulis* (Cardoso-Gustavson et al., 2013). This alkaloid was retained in the extra-floral nectary at high concentrations as well as excreted into EFN at low concentrations. The plant modulated secondary metabolite concentrations to relate differently to herbivores and mutualistic consumers: high concentrations in EFNs protected the gland from herbivores while low (trace) concentrations in EFN had no apparent effect on ants (Cardoso-Gustavson et al., 2013).

Though not reported in EFN, four alkaloids (caffeine, theophylline, cocaine, and atropine) can have significant effects on many aspects of ant physiology and behavior (Cammaerts et al., 2014; **Table 1**). In particular, when ingested, the alkaloids altered locomotion, memory, olfactory perception and reactions to stimuli in the *Myrmica sabuleti* ant model (**Table 1**). Whether any of these or other neuroactive compounds could be components of EFN, and their effects on attending ants at concentrations plausible for EFN, are not known. In this context, it is worth noting that ants are subject to manipulation by other organisms (Hughes, 2012; Grasso et al., 2015). A recent case regards blue butterflies (Lepidoptera: Lycaenidae) whose caterpillars produce a sugary secretion that attracts ants which then defend the larvae from predators. Hojo et al. (2015) found that these secretions are not simply nutritious food, but also affect ant behavior, enhancing their cooperative services.

A striking case of partner manipulation involving the myrmecophyte *Acacia cornigera* and the mutualist ant *Pseudomyrmex ferrugineus* is therefore not surprising (Heil et al., 2014). These ants only feed on the sucrose-free nectar produced by their host plant; the nectar is not attractive to other generalist exploiter ants. Until a few years ago, *Pseudomyrmex ferrugineus* ants were believed to lack invertase (a sucrose hydrolysing enzyme) in their digestive tract, a physiological trait compensated by the plant through secretion of sucrose-free EFN (Heil et al., 2005). However, this "specialization" hides a clear case of partner manipulation by the host plant. In fact, invertase activity is not constitutionally absent in the ant midgut but is inhibited by chitinase (**Table 1**), a dominant EFN protein that has a primary function in defense against nectar-dwelling pathogenic fungi (González-Teuber et al., 2010). Once eclosed, young workers ingest EFN as the first food available. Since this inhibits their invertase, they are forced to continue feeding on host-derived EFN, being unable to digest any other food (Heil et al., 2014). The plant manipulates the digestive physiology of the symbiotic ants to enhance their dependence on host-derived food rewards, thus stabilizing in the partnership and avoiding possible interference by exploiters.

CONCLUDING REMARKS AND FUTURE CHALLENGES

Recent research on nectar-mediated plant–animal interactions highlights that FN and EFN is much more than a sugary reward for animal services. As suggested by Pyke (2016), nectar can now be viewed as a pollinator manipulant rather than simply an attractant or reward (**Figure 2**). Clear effects of nectar-mediated manipulation are known for pollinating insects and are mainly based on secondary metabolites in FN (**Figure 2**). Although detailed studies are only available for caffeine and nicotine (Kessler et al., 2012; Wright et al., 2013; Baracchi et al., 2017), other known psychoactive compounds from plants could also manipulate pollinator behavior but have not yet been investigated in nectar.

The real outcomes of these manipulative strategies are not yet well-understood. Plants enhance recall of a food resource by presenting appropriate concentrations of psychoactive drugs in FN (Wright et al., 2013; Baracchi et al., 2017). This strategy may ensure pollinator fidelity and possibly improve the plant's reproductive success, but experimental evidence is not yet available (**Figure 2**). On the animal side, although improved recall can be positive for efficient foraging activity, it also has a negative counterpart since bees tend to return to the source of caffeinated nectar when it is no longer available (Couvillon et al., 2015) and this may have negative consequences for the pollinator. It seems that "manipulated" pollinators still obtain the benefits of nectar consumption, but in the case of a net negative outcome for animal fitness, manipulation may turn a mutualism into parasitism (Heil, 2015b; Hojo et al., 2015).

The presence of nectar-dwelling microorganisms adds a further level of complexity to these manipulative interactions (**Figure 2**). Microorganisms such as yeasts and bacteria are very common in FN where they are inoculated by pollinators and can be considered a third partnership in nectar-mediated plant–pollinator interactions (Herrera et al., 2009). They are responsible for drastic changes in nectar chemical profile that potentially affect pollinator behavior and foraging choices: they alter the concentrations of specific sugars and amino acids (Canto and Herrera, 2012; de Vega and Herrera, 2013; Pozo et al., 2014; Lenaerts et al., 2016; Vannette and Fukami, 2018) and

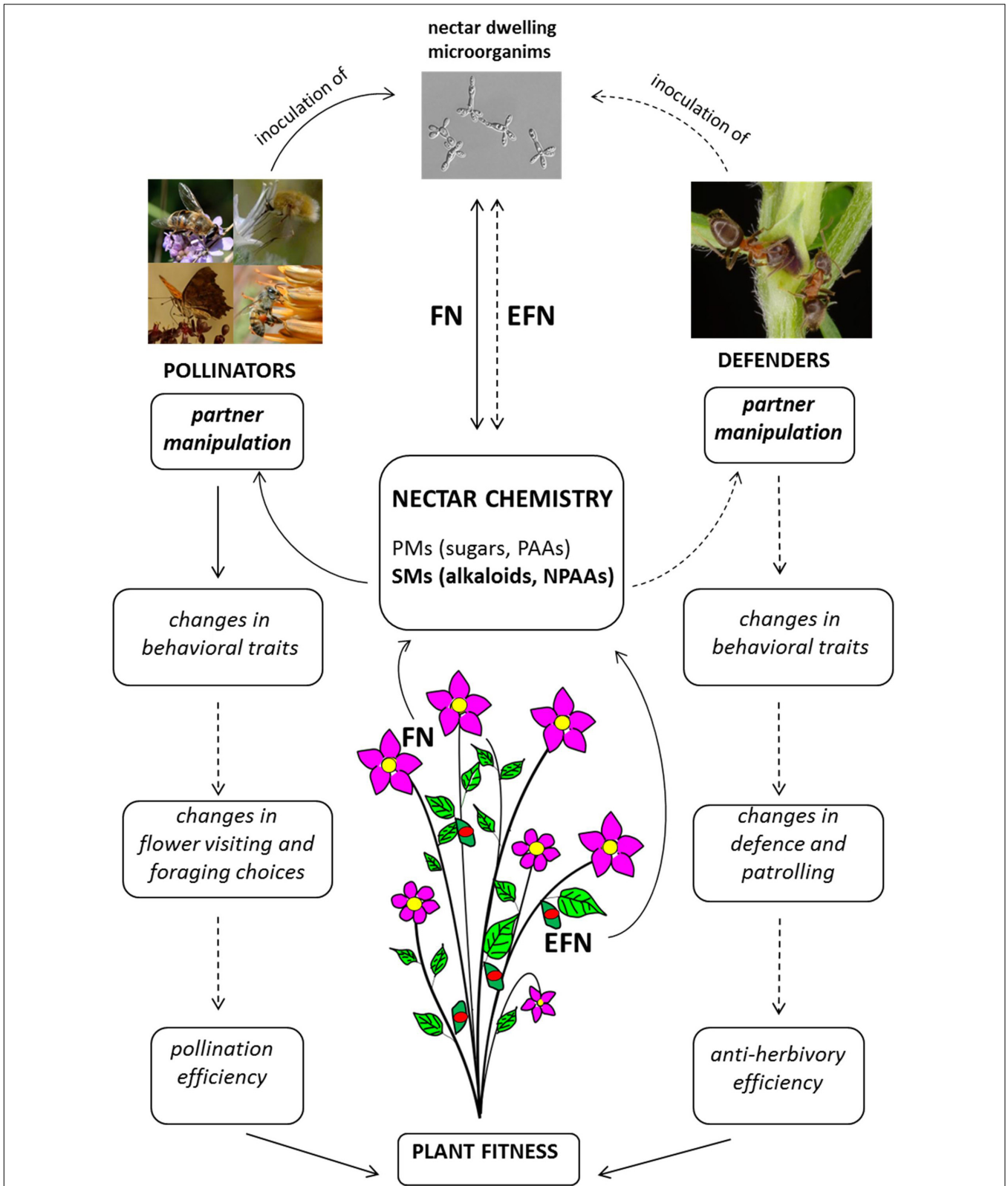

FIGURE 2 | Diagram of nectar-mediated manipulation of pollinators and tending ants. Full lines indicate processes/interactions sustained by scientific evidence; hatched lines indicate processes/interactions for which scientific evidence is not yet available. FN, floral nectar; EFN, extra-floral nectar; PMs, primary metabolites; PAAs, protein amino acids; NPAAs, non-protein amino acids; SMs, secondary metabolites. Picture of nectar-dwelling microorganism (*Metschnikowia gruessii*) reproduced with permission from Carlos M. Herrera.

produce volatile substances that are perceived by pollinators (Raguso, 2009). Interestingly, microorganisms are also able to alter the profile of nectar SMs. For example, they can significantly lower the concentration of nicotine and thus interactions with pollinators, since the effects of secondary compounds are concentration-dependent (Vannette and Fukami, 2016).

Ants are known to transport microorganisms (de Vega and Herrera, 2013) although the presence of the latter in EFN has never been reported.

Another aspect that needs to be considered in reporting complex outcomes of manipulative exploitation in mutualistic relationships is that nectar is a complex mixture of solutes, while experiments on the effects of nectar-specific compounds are often conducted on single molecules, ignoring any synergic or antagonistic effects.

Secondary metabolites in EFN and their possible interactions with tending ants (and other insects) have not been the subject of much research (**Figure 2**). Complexity similar to that of FN-mediated interactions is also likely for EFN but has not yet been investigated (Grasso et al., 2015). Since the targets of indirect defense by mutualism with ants are plant enemies such as herbivores, aggression is an obvious ant behavioral trait that could be manipulated by plants, although other less conspicuous behaviors could also be affected and have significant positive effects (Grasso et al., 2015).

Plants modulating the concentration of SMs in their tissues and secretions evolved strategies to deter herbivores (high concentrations), while attracting and manipulating mutualists (low concentrations) to maximize the benefits they obtained. When such strategies evolved is hard to say. The oldest plant–insect relationship is predation of plants by herbivores and plants underwent natural selection on the basis of chemical defenses (secondary metabolites) evolved against herbivores. When mutualistic insects evolved (defenders and pollinators) they presumably drove plant selection toward optimal (low) concentrations of SMs (and other substances) in secretions they fed on, while plants probably started to manipulate insect behavior pharmacologically, improving their own fitness. Most "modern" mutualist insects (Diptera, Lepidoptera, and Hymenoptera including ants) radiated in the interval 125–90 Mya (i.e., early-middle Cretaceous), simultaneously with angiosperms (Labandeira, 2011). Nectars with SM profiles presumably evolved and diversified in angiosperms and allowed them more efficient interactions with insects, overriding interactions already established by gymnosperms (Nepi et al., 2017).

Concluding, since conflicts also arise in cooperative partnerships, nectar-mediated partner manipulations may be more frequent than previously thought in plant–insect interactions conventionally regarded as mutualistic. This may provide new evidence supporting the idea that elements of coercion/manipulation are not necessarily linked to parasitic habits but may be functional for stabilizing certain insect–plant mutualisms (Heil, 2015b), opening new horizons in the study of coevolutionary pathways involving these dominant organisms.

AUTHOR CONTRIBUTIONS

MN, DG, and SM conceived the idea of the article and wrote the final version of the paper. MN designed the outline and wrote the draft of the paper. DG and SM commented on the draft.

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Differential Effects of Climate Warming on the Nectar Secretion of Early- and Late-Flowering Mediterranean Plants

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Floral nectar is a vital resource for pollinators, thus having a very important role in ecosystem functioning. Ongoing climate warming could have a negative effect on nectar secretion, particularly in the Mediterranean, where a strong temperature rise is expected. In turn, decreased nectar secretion, together with shifts in flowering phenology can disrupt plant–pollinator interactions and consequently affect the entire ecosystem. Under fully controlled conditions, we tested how temperature influenced nectar secretion (through nectar volume, sugar concentration, sugar content, and number of flowers produced) in six Mediterranean plant species flowering from winter to summer (viz. *Asphodelus ramosus*, *Ballota acetabulosa*, *Echium plantagineum*, *Lavandula stoechas*, *Rosmarinus officinalis*, and *Teucrium divaricatum*). We compared the changes in nectar secretion under temperatures expected by the end of the century and estimated the effect of climate warming on nectar secretion of plants flowering in different seasons. We found a significant effect of temperature on nectar secretion, with a negative effect of very high temperatures in all species. Optimal temperatures for nectar secretion were similar to the mean temperatures in the recent past (1958–2001) during the respective flowering time of each species. Increasing temperatures, however, will affect differently the early-flowering (blooming in winter and early spring) and late-flowering species (blooming in late spring and early summer). Temperature rise expected by the end of the century will shift the average temperature beyond the optimal range for flower production and the sugar produced per plant in late-flowering species. Therefore, we expect a future decrease in nectar secretion of late-flowering species, which could reduce the amount of nectar resources available for their pollinators. Early-flowering plants will be less affected (optimal temperatures were not significantly different from the future projected temperatures), and may in some cases even benefit from rising temperatures. However, as many earlier studies have found that early-flowering species are more prone to shifts in phenology, the plant–pollinator interactions could instead become affected in a different manner. Consequently, climate warming will likely have a distinctive effect on both plant and pollinator populations and their interactions across different seasons.

Keywords: climate change, floral nectar, Mediterranean plants, nectar resource, optimal temperature, plant–pollinator interactions, seasonal differences

INTRODUCTION

Global temperatures show an ever-increasing trend (NOAA, 2018), which is expected to have a considerable effect on numerous species, their interactions and the entire ecosystems (Parmesan and Yohe, 2003; Tylianakis et al., 2008; Traill et al., 2010). In the Mediterranean region, the temperature change by the year 2100 is expected to be particularly strong, with up to 1.5–2.4°C (max 3.0°C) increase in winter and 2.3–3.3°C (max 5.5°C) in summer, in comparison to the second half of the twentieth century (Giorgi and Lionello, 2008; Giannakopoulos et al., 2009; IPCC, 2013). Therefore, climate warming is predicted to have a pronounced effect on Mediterranean ecosystems (Sala et al., 2000; Giorgi, 2006; Malcolm et al., 2006).

Temperature rise can affect plant species and entire communities in multiple ways, by imposing, e.g., phenological shifts (Walther, 2003; Gordo and Sanz, 2009; Bock et al., 2014), physiological temperature stress (Scaven and Rafferty, 2013; Bussotti et al., 2014), and disrupted interactions with mutualists (Memmott et al., 2007). Shifts in phenology in response to climate warming have already been widely recorded in many organism groups across the world (Parmesan, 2006; Cleland et al., 2007; Bertin, 2008; Miller-Rushing and Primack, 2008). In plants, the shifts are usually stronger in early-flowering species and less marked in late-flowering plants (Fitter and Fitter, 2002; Walther et al., 2002; Petanidou et al., 2014).

Ambient temperatures directly affect plant physiology. The optimal range of ambient temperatures for photosynthesis in Mediterranean woody plants under experimental conditions is usually around 25–30° (Flexas et al., 2014), but the optimum can also shift according to season (Medlyn et al., 2002) and be somewhat lower under field conditions (Flexas et al., 2014). Temperatures in the Mediterranean maquis (evergreen-sclerophyllous scrub) reach 35–40°C in summer, but leaf temperature can be even up to 55°C under the same conditions (Larcher, 2000). However, photosynthesis can already start progressively diminishing when leaf temperature is between 35 and 40°C (Larcher, 2000). Altogether, plants in the Mediterranean generally grow under suboptimal temperatures in winter (Larcher, 2000) and close to their optimum (Bussotti et al., 2014) or occasionally even at supra-optimal temperatures in summer (Larcher, 2000; Flexas et al., 2014). However, under future climate warming the optimal temperatures in summer might be exceeded more frequently than before (Bussotti et al., 2014).

Temperature also affects plants indirectly through processes dependent on plant photosynthetic capacity, such as flower and nectar production (Southwick, 1984; Burquez and Corbet, 1998). The effect of elevated temperatures on the number of flowers has been found ambiguous, with both increase and reduction in the number of flowers in different species, or with no change at all (Jakobsen and Kristjánsson, 1994; Liu et al., 2012; Scaven and Rafferty, 2013). A strong heat stress during flowering, however, can cause abortion of buds and open flowers and thus reduce their number (Morrison and Stewart, 2002; Wahid et al., 2007; Bykova et al., 2012). Plants can also produce more flowers without any nectar under temperature stress (Petanidou and Smets, 1996; Takkis et al., 2015). Floral nectar volume is unimodally related to temperature and the optimal temperatures generally correspond well to average ambient temperatures during the flowering season (Jakobsen and Kristjánsson, 1994; Petanidou, 2007). Moderately elevated temperatures may increase nectar secretion (Pacini and Nepi, 2007; Nocentini et al., 2013), but strongly elevated temperatures reduce it (Petanidou and Smets, 1996; Scaven and Rafferty, 2013; Takkis et al., 2015). At the same time, nectar sugar concentration is generally less variable and less affected by temperature than nectar volume (Villarreal and Freeman, 1990; Nocentini et al., 2013; Takkis et al., 2015). Altogether, under elevated temperatures, plant overall nectar secretion could be reduced through a combined negative effect of high temperatures on flower and nectar production.

Combined warming-induced changes in phenology and nectar production can alter plant–pollinator interactions through phenological mismatches and reduced nectar resources available for pollinators (Memmott et al., 2007; Hegland et al., 2009; Petanidou et al., 2014). The most likely reason for mismatches are differences in the cues used by the interaction partners, such as temperature or day length (Hughes, 2000; Bertin, 2008; Doi et al., 2008). Mismatches are more likely to occur among spring than summer species, because of stronger phenological shifts early in the season (Doi et al., 2008; Wolkovich et al., 2012; Fründ et al., 2013). The possible changes in nectar resources are still largely unknown. Consequent changes in the interaction networks can have a negative impact on both plants and pollinators, and cause population declines in both groups (Real and Rathcke, 1991; Hegland et al., 2009; Scaven and Rafferty, 2013). Nevertheless, despite the multiple expected changes, plant–pollinator interaction systems are generally considered to be relatively stable and resilient to climate change (Memmott et al., 2004; Devoto et al., 2007; Petanidou et al., 2014).

In addition to the expected temperature rise, current climate change can also alter precipitation patterns. For the Mediterranean region, different projections generally predict decreased amounts of precipitation (Giorgi and Lionello, 2008; Giannakopoulos et al., 2009; IPCC, 2013). However, the differences in precipitation can be great between adjacent localities—even during the recent hottest years on record, the precipitation patterns in the Mediterranean have been complex, with both less and more than average amounts of rainfall in different places (NOAA, 2018). Furthermore, the magnitude of changes can differ between seasons (IPCC, 2013). Due to the varied patterns of precipitation under climate change (Cook and Wolkovich, 2016), its effect on vegetation in any particular region in the future is expected to be also variable.

In this study, we investigate the effect of temperature on the nectar secretion of six common Mediterranean plant species, flowering from winter to summer. By experimentally provoking nectar and flower production under temperature stress in a climate chamber, we compare the effect of the IPCC-projected temperature rise on the early- and late-flowering species. We expect to find evidence of the negative effect of strongly elevated temperatures on nectar and flower production. We hypothesize that nectar secretion in late-flowering

species will be more negatively affected by the predicted climate warming than that of the early-flowering species due to the already very high temperatures characterizing the Mediterranean summer. In the case of different responses of early- and late-flowering species, in combination with the expected phenology changes found in many earlier studies, the effect of climate warming on plant communities, their pollinators, and plant–pollinator interaction networks could have distinctive consequences early and late in the season.

MATERIALS AND METHODS

Focal Species

We tested the effect of temperature on nectar secretion of six native Mediterranean species that are good nectar producers with flowering periods from winter to summer. The species were (in the order of flowering; **Figure 1**): *Rosmarinus officinalis* L. (Lamiaceae), *Asphodelus ramosus* L. (Asphodelaceae), *Lavandula stoechas* L. (Lamiaceae), *Echium plantagineum* L. (Boraginaceae), *Ballota acetabulosa* (L.) Benth. (Lamiaceae), and *Teucrium divaricatum* Sieber ex Heldr. (Lamiaceae). All tested species produce relatively large quantities of nectar and are important resources for different pollinators, including honeybees (Herrera, 1988; Petanidou and Smets, 1995; Potts et al., 2006; Keasar et al., 2008; Dauber et al., 2010; Petanidou et al., unpublished data).

Full-grown plants of *R. officinalis* were obtained from a garden center. *Asphodelus ramosus*, *L. stoechas,* and *T. divaricatum* were collected as entire plants from natural populations on Lesvos Island in October 2013, potted and maintained outdoors until the start of the experiment. *Ballota acetabulosa* and *E. plantagineum* were grown from seeds collected in the wild at the I. & A. Diomedes Botanical Garden of Athens University, Athens, and on Lesvos Island, Greece, respectively. In the case of *B. acetabulosa*, we obtained a subset of seeds collected from *c.* 100 individual plants. The *E. plantagineum* seeds were collected from 30 plants in one population. Plants grown from seeds were germinated in Petri dishes, potted as seedlings and grown outdoors until flowering.

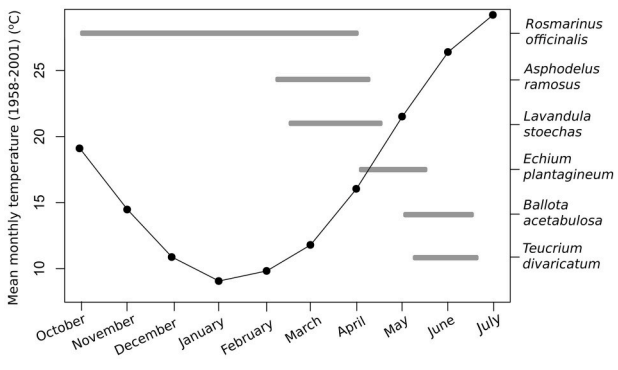

FIGURE 1 | Mean monthly temperatures (1958–2001) and the flowering periods of the six study species in the Aegean region.

Experiment Design

The temperature response of all species was tested in potted plants in an indoor climate chamber (Walk-in GRW-20 CMP 3/TBLIN, CDR Chryssagis™) during their natural time of flowering in 2014 or 2015. We tested the effect of a wide range of temperatures on plant nectar secretion, aiming to obtain a relatively full response curve for each trait. The selected temperatures were centered around the long-term monthly average temperatures at the time of flowering of each species in the recent past (1958–2001, Elefsis weather station, Athens, Greece). Temperature was increased to at least 3 degrees above the expected temperature maxima according to climate change projections (IPCC, 2013) for that particular season to ensure the stability of the observed trend, or until the flowering finished. We increased the temperature in consistent increments every 3 days. By applying an incremental temperature rise, we allowed for the temperature hardening (acclimatization to higher temperatures) in plants, similarly to natural conditions (Larcher, 2000), which allows us to find the true temperature limitations of these species. Night temperatures were always kept 6°C lower than the day temperatures, simulating natural conditions. The day/night light regime followed approximately the natural diurnal cycles appropriate for the flowering time of each species. Plants were grown under a mixture of plant growth fluorescent lamps (Gro-lux) and low-pressure sodium lamps, with a total light intensity of *c.* 800 μmol m^{-2} s^{-1} (*c.* 43,000 lx) over the waveband 400–700 nm. Relative air humidity was kept constant throughout the experiments, at 60 ± 5% at daytime and 80 ± 5% at night. All plants were watered on Day 1 of each temperature step. For details on the experimental conditions of each species, see **Table 1**.

In addition to the experimental treatments in the climate chamber, we followed control groups of five of the study species (**Table 1**), to be able to separate the effect of the manipulated temperatures from the natural changes occurring during the flowering period (the effect of time). Plants in a similar flowering stage were randomly divided between the experimental and control group. The controls were in most cases conducted parallel to the experimental treatments and ended when the plants had a comparable number of open flowers as in the experimental group. Only in the case of *A. ramosus*, the control was conducted a year later than the experimental treatment. It was carried out in the climate chamber under controlled conditions with all other settings the same as in the main experiment, but with the temperature kept constant (**Table 1**).

The controls for *R. officinalis*, *E. plantagineum*, *B. acetabulosa,* and *T. divaricatum* plants were conducted simultaneously with the experimental group treatments, but outdoors under naturally varying conditions. The plants were placed in full sunlight under tulle cages to prevent visitation by pollinators. The control data for *R. officinalis* could not be used for the analyses. During the first two sampling periods, there was an unexpected cold spell (near-freezing temperatures) and the plants produced almost no nectar. During the last two sampling periods, the nectar was diluted due to rainfall and was therefore unsuitable for analysis. Consequently, there were

TABLE 1 | Experimental conditions of the six study species.

	Rosmarinus officinalis[a,b]	*Asphodelus ramosus*[c]	*Lavandula stoechas*	*Echium plantagineum*[b,d]	*Ballota acetabulosa*[e]	*Teucrium divaricatum*[e]
EXPERIMENTAL GROUP						
Flowering time (month of peak flowering)	October–April (midpoint January)	March	April	May	June	June
Date	06.–29.01.2015	12.03.–28.03. 2014	30.03.–19.04.2014	05.05.–25.05. 2015	24.05.–17.06. 2014	24.05.–17.06.2014
Day temperatures (°C)	7–34	12–22	16–28	14.5–38.5	20–41	20–41
24 h average temperatures (°C)	3.5–30.5	8.8–18.8	12.8–24.8	12–36	17.5–38.5	17.5–38.5
Temperature increments (°C)	4	2	2	4	3	3
Number of steps	8	6	7	7	8	8
Light/dark (h)	10/14	11/13	11/13	14/10	14/10	14/10
Number of plants	19	12	20	15	15 + 1	15 + 11
CONTROL GROUP						
Placement	Outdoors	Climate chamber	–	Outdoors	Outdoors	Outdoors
Date	06.–29.01.2015	03.–24.03. 2015		05.05.–25.05. 2015	24.05.–08.07.2014	24.05.–08.07.2014
Day temperature (°C)	Failed (see text for details)	15		–	–	–
24 h average temperature (°C)		11.8		–	–	–
Number of steps		6		7	15	15
Light/dark (h)		11/13		–	–	–
Number of plants	9	11		6	6	6 + 6

[a] *First increment was 3°C due to technical limitations of the climate chamber.*

[b] *Plants were treated twice during the experiment with the solution of Caster 20SL insecticide to treat a minor parasite infestation.*

[c] *Due to two general power cuts (lasting several hours but with a prior notice given) the control group experiment had to be stopped twice and the plants were taken outdoors for the time of the blackout to maintain the dark/light regime. The temperature outdoors at the time was similar to that in the chamber. After resuming the experiment, the plants were again given time to adjust to the chamber to ensure equal sampling conditions. As a result, in two cases the time between two measurements was 5 days instead of the usual three. The interruptions did not have any detectable influence on the patterns of flowering and nectar production.*

[d] *Plants were additionally watered, if necessary, on Day 3 after nectar sampling to retain soil moisture under extremely high temperatures.*

[e] *Some of the original plants of were replaced when they reached the end of their flowering period, in order to have an equal number of test plants at each temperature step. e.g., one plant was replaced in the case of B. acetabulosa, so that each step would have 15 plants (number of plants: 15 + 1).*

too few sampling periods (four out of eight) for reliable use.

Nectar Sampling and the Number of Flowers

Nectar sampling was conducted uniformly in all species and both in the experimental and control groups. Sampling was performed on Day 3 of every temperature step, starting at 12:30. Nectar was sampled from flowers during their first day of anthesis. To ensure that we only sampled fresh flowers, all flowers were removed on Day 2, 24 h prior to sampling. In the case of *A. ramosus*, the flowers were marked instead of removed, to avoid excessive damage to the plant. Nectar was sampled from three randomly taken flowers per plant using Drummond microcaps® (0.25–10 µl, depending on the size and nectar quantity of the flowers of each plant). Nectar sugar concentration was measured with hand refractometers calibrated for small nectar volumes (Bellingham and Stanley LTD, Tunbridge Wells). Nectar sugar content per flower was calculated based on the measured nectar volume and sugar concentration (volume × concentration × density), with sugar solution density obtained from available tables (page 278 in Dafni et al., 2005). After sampling in Day 3, all new flowers produced during the previous 24 h were counted and removed (or marked). Sugar content per plant was calculated based on the average sugar content per flower and the number of open flowers per plant during Day 3 of each temperature step.

Climate Data

We used the long-term (1958–2001) average monthly temperatures from the Elefsis weather station, Athens, Greece, to compare the optimal temperatures to the average climate conditions in the recent past. The average temperatures during the flowering time of our study species in the region were the following: January 9.2°C, March 11.9°C, April 15.9°C, May 21.3°C, and June 26.2°C (**Figure 1**). We used the peak flowering

time in the nature to make comparisons for each species with past and future temperatures within that month (Petanidou, 1991; Petanidou et al., unpublished data). In the case of *R. officinalis*, we used January for the experiment and comparisons, as it is the approximate mid-point of the plant's long flowering period from autumn to spring (Castro-Díez and Montserrat-Marti, 1998; Keasar et al., 2008).

Future projections for each month considered in the analysis for the Mediterranean region were obtained from the IPCC reports (IPCC, 2007, 2013). We used the projections of the RCP4.5 stabilization scenario (IPCC, 2013), which predicts a 1.5–2.4°C (25th−75th percentiles; max 3.0°C) warming for the winter months (December–February), and a 2.3–3.3°C (max 5.5°C) warming for the summer months (June–August) in the Mediterranean region for the period 2081–2100, compared to the reference period 1986–2005. Since the exact data on spring months were not given for the RCP4.5 scenario, then for this period (March–May) we used the projections of the A1B scenario (IPCC, 2007) that predicts a 2.1–2.7°C (max 3.7°C) warming for the period 2080–2099 compared to the reference period 1980–1999. Both scenarios consider stabilizing greenhouse gas emissions and are comparable in their projections (IPCC, 2007, 2013).

Data Analysis

We tested the effect of temperature on five traits measured per day (Day 3 of each temperature step): (1) nectar volume per flower, (2) nectar sugar concentration per flower, (3) nectar sugar content per flower, (4) nectar sugar content per plant, and (5) the number of flowers per plant. For the first three traits we used average values per plant, i.e., the mean value of the three sampled flowers. When nectar volume in a flower was too small to measure nectar sugar concentration, we inferred this value based on the other flowers sampled from the same plant. This calculation was done in the case of *R. officinalis* for 27/358 flowers, in *L. stoechas* for 43/358 flowers, in *B. acetabulosa* for 11/341 flowers and in *T. divaricatum* for 2/325 flowers.

Nectar volume, sugar content per flower, sugar content per plant and the number of flowers per plant were tested for normality using Shapiro–Wilk test and log-transformed. The sugar concentration data were logit-transformed to remove the constraints of percentage data. All response and explanatory variables were standardized (mean = 0, $SD = 1$) in order to compare the six species. In the case of *T. divaricatum*, we tested for the potential differences between the original and replacement plants (see **Table 1**) but no significant differences were found (for more details see Takkis et al., 2015).

Prior to the main analysis, we examined whether the flower and nectar production trends in the experimental groups were significantly affected by the manipulated temperatures and not caused solely by the natural changes throughout the flowering period. The aim of this analysis was to validate the use of the data only from the experimental group for the following analyses. In order to separate the true effect of manipulated temperatures from the effect of time and natural changes through the flowering period, we compared the experimental and control groups in the four species with reliable control data (**Table 1**). The comparison is based on the assumption that plants in the experimental and control group respond to time uniformly, but in the experimental group, there is an added effect of elevated temperatures. Hence, a significant interaction between time and treatment in the models would indicate a significant difference between the groups, caused by the elevated temperatures in the experimental group (**Figure 2**).

For this purpose, we added treatment group (experimental or control) as a binary variable into the linear mixed models (LMM) analysing each trait and used the "time × treatment group" interaction to detect possible differences between the two groups. Separate models were compiled for each of the four species, testing the simple and squared terms of each trait and using plant ID as a random factor in the analyses. Additionally, a combined model for all four species was built, using plant ID nested within the species as a random factor. We tested both simple and quadratic effect of the time and compared which of the models had a better fit based on their AIC values. To be able to compare different trait values among species and different time periods (different length of flowering periods of different species and also outdoors controls sometimes lasted longer than the main experiment), we standardized the parameters (mean = 0, $SD = 1$) when necessary.

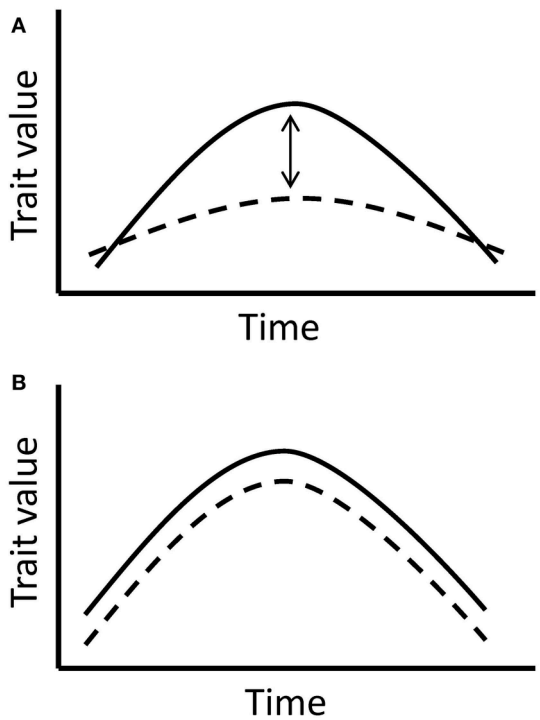

FIGURE 2 | The models compare trait responses to time in the experimental and control group. **(A)** If the interaction between time and treatment group (experimental or control) is significant, it implies a true significant effect of manipulated temperatures indoors, since the effect of time is expected to be similar in all tested plants. **(B)** If the interaction between time and treatment group is non-significant, it indicates the lack of a significant temperature effect in the experimental group and shows a more prevalent effect of time on the trait.

For the main analysis, we first divided the six species into two groups–(1) the species flowering in winter and early spring (hereafter early-flowering)–*R. officinalis*, *A. ramosus,* and *L. stoechas*, flowering between January and April, and (2) the species flowering in late-spring and summer (hereafter late-flowering)–*E. plantagineum*, *B. acetabulosa*, and *T. divaricatum*, flowering from May to June (Petanidou et al., 1995, 2014). The early- and late-flowering species' phenology often exhibits differential responses to climate warming (Petanidou et al., 1995, 2014; Fitter and Fitter, 2002; Bertin, 2008). Therefore, we could also expect differences between the two groups in other traits, such as nectar production, in response to warming. We tested whether these two groups respond differently to manipulated temperatures, using the interaction of temperature (simple and quadratic effect) and species group (early- or late-flowering) in the linear mixed models (LMM). We used plant ID nested within species as random effects.

Secondly, we fitted LMM models for each species separately (with plant ID as a random factor), to calculate the optimal temperature range for each trait in each species. We calculated the temperature optimum based on the model maximal values, considering 5% of the highest trait values as the optimal region and the corresponding temperature range as the optimal temperature range for the given trait (**Figure 3**). In order to understand the response of the early- and late- flowering species, we compared the optimal ranges to the average monthly long-term temperatures in the study region in the recent past (1958–2001, Elefsis weather station, Athens, Greece) and the temperature changes projected for 2100 (IPCC, 2007, 2013) to estimate the species ability to withstand future climate change. We used paired *t*-tests to see if the optimal temperatures of the early- and late-flowering species differ significantly from the past monthly average temperatures and from those predicted for 2100. The tests were conducted for all traits, except for sugar concentration, which in several species had a linear, not unimodal relationship to temperature and therefore did not allow for the optimal range to be calculated in several species.

In addition, we tested the effect of temperature (simple and quadratic effect) on the proportion of empty flowers (flowers producing no nectar; calculation based on the three sampled flowers) in *R. officinalis*, *L. stoechas*, and *B. acetabulosa*, which had numerous flowers with no nectar. The rest of the species did not have any empty flowers or had very few (in the case of *T. divaricatum*). We used zero-inflated generalized linear mixed models (ZI-GLMM) with negative binomial error distribution for the analysis, with plant ID nested within species as a random factor.

All statistical analyses were conducted in R 3.4.2 (R Core Team, 2017) in the RStudio 1.1.383 environment (R Studio Team, 2016). LMM models were tested using the function *lmer* in the *lme4* package (Bates et al., 2015). Additional *p*-values were calculated with the package *lmerTest* (Kuznetsova et al., 2017). Marginal and conditional coefficients of determination (R^2m and R^2c) for the LMM models were calculated with the function *r.squaredGLMM* in the package *MuMIn* (Bartoń, 2016). ZI-GLMM models were built with the function *glmmadmb* in the package *glmmADMB* (Fournier et al., 2012; Skaug et al., 2015). Figure S1 was prepared with the function *ggplot* in the *ggplot2* package (Wickham, 2009), using a smoothing function to plot the relationships.

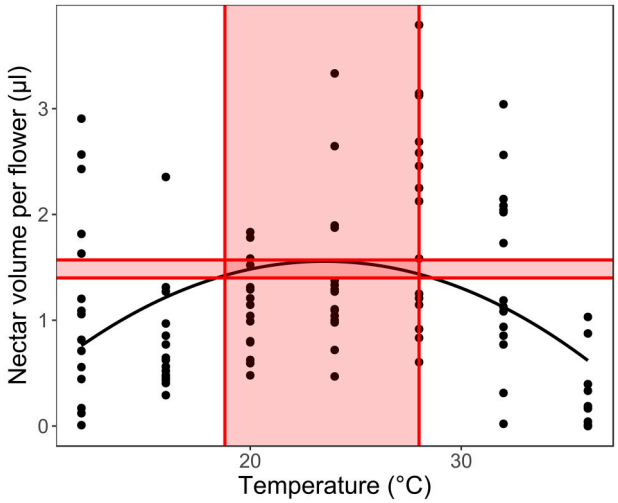

FIGURE 3 | Calculating the optimal temperature range for each trait in each species. We used 5% of the measured trait value range below the calculated optimum as the optimal production range (shaded area between the horizontal lines) to calculate the optimal temperature range for the given trait (shaded temperature values between the vertical lines).

RESULTS

The results of the control models separating the effect of temperature from that of time based on the comparison with the control groups in four species (**Table 2**) indicated that nectar volume per flower and sugar content per flower and per plant were significantly affected by manipulated temperatures in the experimental group. This was true in most species separately and in all species combined. Sugar concentration per flower was not affected by temperature in any of the species separately, but showed a significant effect when the species were combined. At the same time, the number of flowers showed a significant response to temperature in the three species separately, but not when the species were combined.

The test indicated that there was a significant effect of manipulated temperatures on the number of flowers and nectar traits (particularly for nectar volume, sugar content per flower and per plant). The effect was relatively consistent across species, indicating therefore that it can be extrapolated with high likelihood to those species where controls were not performed or failed (**Table 1**). Thus, we conclude that the results of the experimental groups can be used independently to study the effect of temperature across all study species in the following main analyses. In

the case of nectar sugar concentration and the number of flowers, the results were more ambiguous and variable across species, and should be used and interpreted with some caution.

In the main LMM analysis, we found that all tested traits were related to temperature either unimodally (for nectar volume, sugar content per flower and per plant, and the number of flowers) or linearly (sugar concentration per flower). However, early- and late-flowering species responded differently to temperature in most traits, except in nectar volume per flower (**Table 3**).

The optimal temperature ranges showed expected differences among species, but also among different traits within the species (**Table 4**). Optimal temperatures for nectar volume and flower sugar content followed roughly the monthly average temperatures (**Figures 4A–C**), whereas the optimal temperature for the number of flowers was more uniform in all species (**Figure 4D**). Sugar concentration demonstrated the largest variation in trends among species (linear and unimodal, negative, and positive; **Table 4**).

The *t*-tests showed that for all traits the long-term average temperatures in the recent past are comparable to the optimal temperatures for nectar volume per flower, sugar content per flower and per plant and the number of flowers per plant (**Table 5**, Supplementary Material). In the case of nectar volume per flower, the future projected temperatures are also not significantly different from the optimal temperatures. In the case of early-flowering species, non-significant results were also found for all other traits, whereas the tests were significant or marginally significant for late-flowering species, indicating stronger differences between the optimal and projected temperatures in the future in this plant group (**Figure 4**, **Table 5**).

The proportion of empty flowers in *R. officinalis*, *L. stoechas,* and *B. acetabulosa* had a negative unimodal response to temperature, indicating a considerably higher production of

TABLE 2 | Difference in the effect of time (simple and quadratic effect, "time" and "time^2") on nectar traits and the number of flowers between the experimental and control groups ("group") in the four species for with reliable control data.

Trait	Interaction terms of the models	*Asphodelus ramosus*		*Echium plantagineum*		*Ballota acetabulosa*		*Teucrium divaricatum*		Species combined	
		t	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
Nectar volume per flower	Time × group	3.119	**	−4.900	***	−0.757		0.481		**−0.362**	
	Time^2 × group	0.968		4.881	***	3.543	***	−2.540	*	**3.715**	***
Sugar concentration per flower	Time × group	−1.667		1.611		0.310		−0.606		**2.334**	*
	Time^2 × group	−0.501		−0.458		−1.841		1.868			
Sugar content per flower	Time × group	3.529	**	−4.988	***	0.554		1.058		**2.012**	*
	Time^2 × group	1.417		5.786	***	3.880	**	−0.997		**4.225**	***
Sugar content per plant	Time × group	2.376	*	−4.030	***	−1.209		−1.041		**0.349**	
	Time^2 × group	1.008		4.695	***	3.007	**	−0.066		**2.644**	**
Number of flowers per plant	Time × group	0.368		−2.075	*	−3.824	***	−0.418		**−0.059**	
	Time^2 × group	−0.823		2.182	*	1.027		2.679	**	**1.312**	

*Only interaction terms are presented here from the model full results and only for the best models (with or without the quadratic effect), according to model AIC values. The analysis with the four species combined is given at the right column in bold. *0.05–0.01, **0.01–0.001, *** <0.001.*

TABLE 3 | Differential dependence of nectar and flower traits on temperature and flowering groups (viz. early- and late-flowering species) in all six species.

	Nectar volume per flower	Sugar concentration per flower	Sugar content per flower	Sugar content per plant	Number of flowers per plant
Intercept	4.407***	−0.179ns	5.343***	5.779***	4.698***
Temperature	−3.788***	3.850***	−4.989***	0.067*ns*	7.523***
Temperature^2	−7.719***		0.494***	−10.380***	−10.831***
Flowering group	1.027ns	−0.991ns	−9.667ns	0.640ns	1.326ns
Temperature × flowering group	0.577ns	−6.889***	−2.31*	−8.322***	−15.524***
Temperature^2 × flowering group	−0.737ns		−1.227ns	−0.829ns	0.663ns
R^2m	0.16	0.07	0.28	0.33	0.34
R^2c	0.34	0.29	0.45	0.46	0.56

*Given numbers are t-values along with test significance. R^2m, marginal coefficient of determination, denotes the variation explained by model fixed factors and R^2c, conditional coefficient of determination, denotes the variation explained by both fixed and random factors together. Only best model results are presented (with or without the quadratic effect), according to model AIC values. *0.05–0.01, *** <0.001, ns, non-significant.*

TABLE 4 | Optimal temperatures and optimal ranges for nectar secretion and flower production (24-h average temperatures).

Species	Nectar volume per flower	Sugar concentration per flower	Sugar content per flower	Sugar content per plant	Number of flowers per plant
Rosmarinus officinalis	15.7 (11.1–20.3)	–	16.0 (12.7–19.4)	17.7 (15.5–19.8)	19.7 (16.5–22.9)
	⋂***	/***	⋂***	⋂***	⋂***
Asphodelus ramosus	12.4 (9.4–15.4)	–	12.5 (9.7–15.3)	14.7 (13.0–16.4)	15.9 (13.9–18.0)
	⋂***	/***	⋂***	⋂***	⋂***
Lavandula stoechas	16.4 (12.5–20.2)	15.6 (12.5–18.7)	–	15.3 (13.5–17.0)	18.9 (17.0–20.9)
	⋂ns	⋂**	***	⋂***	⋂***
Echium plantagineum	23.1 (18.8–27.4)	9.4 (0.4–18.4)[a]	20.8 (17.8–23.7)	20.8 (18.6–23.0)	18.7 (11.6–25.8)
	⋂***	⋂***	⋂***	⋂***	⋂***
Ballota acetabulosa	25.9 (22.3–29.6)	26.8 (18.5–35.0)	25.7 (23.0–28.3)	25.4 (23.6–27.2)	24.7 (22.1–27.2)
	⋂***	⋃***	⋂***	⋂***	⋂***
Teucrium divaricatum	30.9 (23.8–37.9)	–	28.7 (23.2–34.1)	22.5 (20.0–25.0)	20.2 (17.5–22.9)
	⋂**	\ns	⋂***	⋂***	⋂***

*Linear associations are marked with "/" (positive) and (negative); unimodal associations with "⋂" (positive) and "⋃" (negative). **0.01–0.001, *** <0.001, ns, non-significant.*
[a]*The abnormally low, and likely incorrect value is probably caused by the nearly linear relationship of the trait.*

TABLE 5 | Comparison of the optimal temperatures within a species group (early- and late-flowering) to the average monthly temperatures in the recent past and future projections for the year 2100 (IPCC, 2013) in the month of flowering of each species (results of paired *t*-tests).

Trait	Early-flowering species			Late-flowering species		
	d.f.	*t*	*p*	*d.f.*	*t*	*p*
NECTAR VOLUME PER FLOWER						
1958–2001 monthly average	2	1.250	ns	2	1.426	ns
50% projections	2	0.109	ns	2	−0.416	ns
75% projections	2	−0.048	ns	2	−0.715	ns
Maximal projections	2	−0.433	ns	2	−1.868	ns
SUGAR CONTENT PER FLOWER						
1958–2001 monthly average	1	1.194	ns	2	0.500	ns
50% projections	1	0.455	ns	2	−2.304	ns
75% projections	1	0.354	ns	2	−2.837	ns
Maximal projections	1	0.102	ns	2	−5.047	*
SUGAR CONTENT PER PLANT						
1958–2001 monthly average	2	1.344	ns	2	−1.633	ns
50% projections	2	0.468	ns	2	−3.932	.
75% projections	2	0.352	ns	2	−4.158	.
Maximal projections	2	0.035	ns	2	−4.530	*
NUMBER OF FLOWERS PER PLANT						
1958–2001 monthly average	2	2.481	ns	2	−2.486	ns
50% projections	2	1.436	ns	2	−4.316	*
75% projections	2	1.319	ns	2	−4.541	*
Maximal projections	2	0.916	ns	2	−5.074	*

*·0.1–0.05, *0.05–0.01, ns, non-significant.*

empty flowers at higher temperatures, but also a slightly higher occurrence at the lowest temperatures (**Table 6**).

DISCUSSION

We found that the progressing climate warming could alter nectar and flower production in different Mediterranean species by the end of this century. The optimal temperatures for nectar secretion in most traits were close to the long-term average temperatures in the recent past (**Figure 4**, **Table 5**), confirming the plants' adaptation to past climate conditions (Jakobsen and Kristjánsson, 1994; Bussotti et al., 2014). In the case of *R. officinalis*, the optima were higher than the past average temperatures in January (the mid-point of its flowering period), indicating the species' adaptation to a wider range of temperatures, which matches its long flowering period from

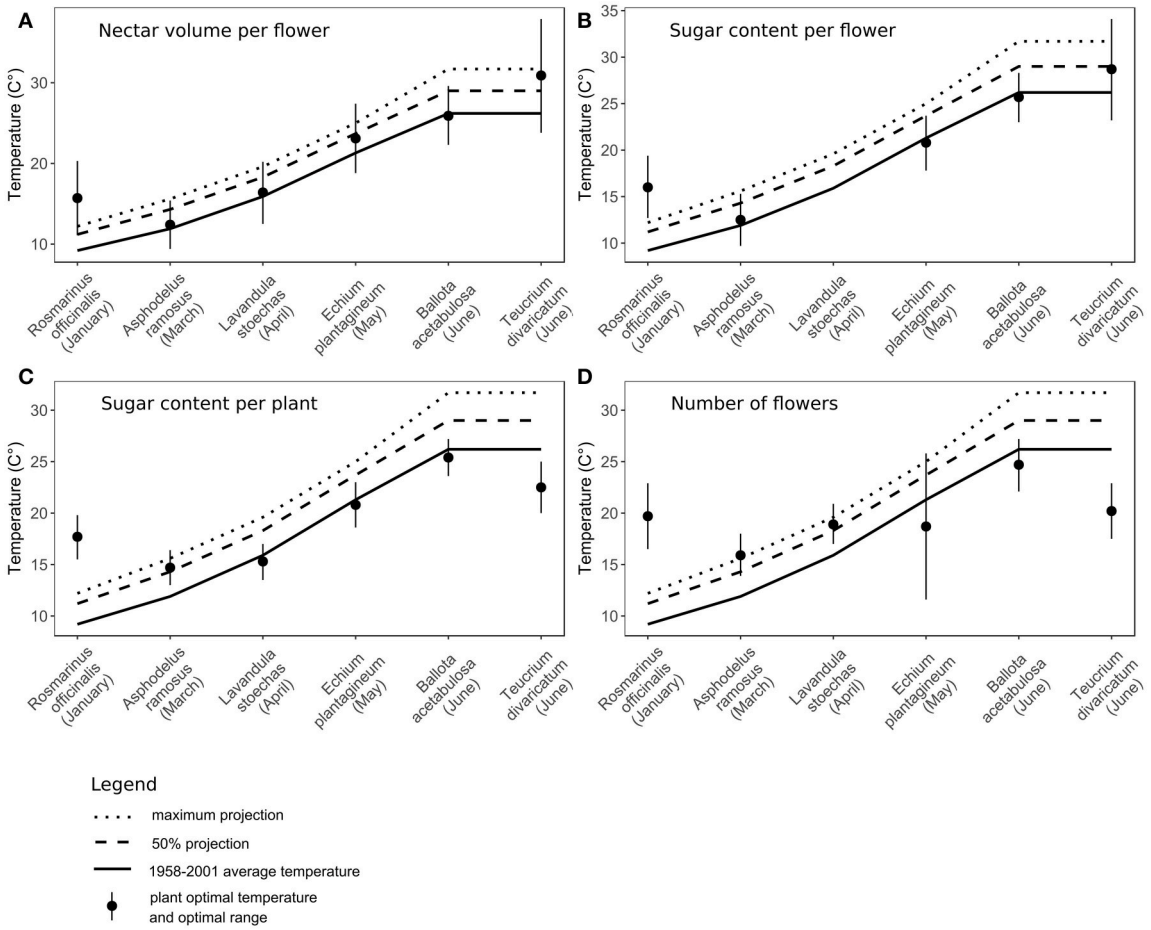

FIGURE 4 | Comparison of optimal temperatures to past monthly average temperatures and future projections by 2100 (IPCC, 2007, 2013) in six species. Flowering month of each species is noted in the parentheses. **(A)** Nectar volume per flower, **(B)** sugar content per flower, **(C)** sugar content per plant, **(D)** the number of flowers per plant. For *Lavandula stoechas*, sugar content per flower (graph B) had a linear relationship to temperature, therefore the optimal temperature could not be calculated.

autumn to spring. Extremely high experimental temperatures reduced nectar secretion in all species through reduced volumes, sugar content, number of flowers, and a greater proportion of empty flowers in some species, corresponding to earlier studies in different plant species (Petanidou and Smets, 1996; Keasar et al., 2008; Scaven and Rafferty, 2013). Admittedly, within the frame of the future temperature rise by 2100 (IPCC, 2013), the actual effect of warming on nectar secretion will likely be less pronounced and differing between seasons.

The effect of expected warming was significantly different on early- and late-flowering species nectar secretion. Both early- and late-flowering species responded similarly regarding nectar volume per flower, which was not compromised by the rising temperatures projected for 2100 (**Table 4**). However, nectar volume could be more susceptible to possible additional reduction in soil humidity coinciding with rising temperatures than to temperature rise *per se* (Villarreal and Freeman, 1990; Petanidou, 2007; IPCC, 2013). In the rest of the traits, the early- and late-flowering species differed in their responses (**Tables 2**, **4**). Sugar concentration showed a positive response to elevated temperatures in early-flowering species and negative response in the case of late-flowering species. However, the individual responses were very variable between species (**Table 4**) and the pure effect of temperature uncoupled from time was somewhat questionable (**Table 3**), therefore the effect of concentration changes within the frame of the future warming is difficult to interpret. In the case of sugar content per flower and per plant, and the number of flowers, for the early-flowering species the optimal temperatures will not be significantly surpassed under future warming. In the late-flowering species, however, nectar sugar content per flower and per plant could be marginally affected by the rising temperatures by the end of the century. Elevated temperatures could compromise nectar sugar content in late-flowering species at least occasionally during the hotter parts of the day or during heat wave events (Larcher, 2000; Bussotti et al., 2014), which are predicted to become more frequent in the future (Giannakopoulos et al., 2009; Rahmstorf and Coumou, 2012; IPCC, 2013). The greatest negative impact

TABLE 6 | The percentage of empty flowers in *Rosmarinus officinalis*, *Lavandula stoechas* and *Ballota acetabulosa* in relation to temperature.

	Estimate	*SE*	*z*	*p*
Intercept	3.843	0.092	41.81	***
Temperature	0.087	0.033	2.61	**
Temperature^2	0.133	0.040	3.34	***

*Model standard errors (SE) and z-values are presented in the table. **0.01–0.001, *** <0.001.*

of elevated temperatures on late-flowering species will probably be through the number of flowers, at least in multi-inflorescence species, such as most of our study species (except for *A. ramosus*), which reduce their number of flowers under heat stress (**Table 2**; Liu et al., 2012). Reduced number of flowers can in turn strongly affect the whole plant's nectar secretion and thus the available resources for pollinators.

Seasonal differences of the effect of climate warming on nectar secretion could be expected in the future. Early-flowering species' nectar secretion might benefit from the temperature rise, whereas late-flowering species could be moderately disadvantaged. Some species flowering very early in the year could encounter temperatures closer to their optimum than the past ones (Llorens et al., 2003) and produce higher amounts of nectar and sugar. For other early-flowering species, the optimal temperatures might be surpassed to some degree, but not significantly (**Figure 4**, **Table 5**). Also, the phenology of early-flowering species is found to be relatively flexible, so under warming they can shift their phenology to remain within their optimal temperature range (Post and Stenseth, 1999; Fitter and Fitter, 2002; Walther et al., 2002). At the same time, the conditions can become increasingly harder for species flowering toward summer. Temperatures in the Mediterranean are expected to rise in the future more rapidly in summer than in any other season (Giorgi and Lionello, 2008; IPCC, 2013) and can surpass the optimal temperatures for nectar and sugar production. Mediterranean plants are generally well adapted to high temperatures and summer drought (Gratani and Varone, 2004; Petanidou, 2007; Miranda et al., 2011; Nuru et al., 2012). However, late-flowering species are already close to or beyond the optimal temperatures for photosynthesis (Larcher, 2000; Bussotti et al., 2014; Flexas et al., 2014), which determines the resources available for flower and nectar production (Southwick, 1984; Burquez and Corbet, 1998; Pacini et al., 2003). Therefore, any increase in temperature can decrease the functioning of late-flowering species more easily compared to early-flowering ones. The optimal temperatures for flower production in summer are already now slightly exceeded (although non-significantly) by the monthly average temperatures and will be significantly surpassed in the future (**Figure 4**, **Table 5**), threatening plants with decreased flower production and reduced overall nectar production.

The potential effect of altered resource availability on pollinators can likewise be different early- and late in the season. Early-flying species would probably not be directly affected by reduced quantity or quality of nectar. However, they could be faced with plant phenology shifts often found in early-flowering species, which can indirectly alter the amount of nectar resources available (Bertin, 2008; Wolkovich et al., 2012; Petanidou et al., 2014). Early-flowering plants, at the same time, can lose a number of pollinators due to phenology mismatches and receive lower pollination service as a result (Petanidou et al., 2014). Pollinators flying later in season will probably not be affected by plant phenology shifts (Bertin, 2008; Petanidou et al., 2014), but might need to cope with moderately reduced amounts of nectar, at least during heatwaves or hotter periods of the day. Altogether, altered plant–pollinator interactions could have distinctive effects on both plant and pollinator populations in different seasons.

It is important to note that the effect of climate change on plants is not limited to temperature, but also includes other climatic variables, such as precipitation (Giannakopoulos et al., 2009; Coumou and Rahmstorf, 2012; IPCC, 2013; Petanidou et al., 2018). Altered rainfall patterns can either enhance or alleviate the effects of elevated temperatures (Bussotti et al., 2014; Cook and Wolkovich, 2016). Changes in precipitation under climate warming are highly variable and dependent on local conditions (NOAA, 2018). Therefore, in this study we limited our work only on testing the effect of temperature rise on plants, to discern the singular effect of temperature rise on plants, uncoupled from potential precipitation changes. We certainly acknowledge the possible additional effect of changed rainfall patterns on plants and their nectar secretion (Villarreal and Freeman, 1990; Carroll et al., 2001; Petanidou, 2007), which affects the overall impact of climate change on plant nectar production and plant–pollinator interactions (Petanidou et al., 2018).

It is possible that during the next century, plants will be able to adapt to some degree to climate warming (Parmesan, 2006). Plants are able to adjust their physiology (such as photosynthetic optima) through the annual temperature changes (Medlyn et al., 2002) or elevational differences (Fryer and Ledig, 1972). In fact, both plastic (Nicotra et al., 2010) and rapid evolutionary responses to climate change have been recorded in plants (Jump and Peñuelas, 2005). However, it is hard to predict how much the adaptational shifts could mitigate the negative effects of warming on flower and nectar production. Conditions in the Mediterranean region in summer are already very difficult for plants (Larcher, 2000; Bussotti et al., 2014) and the potential for adaptation to even harsher conditions, on a relatively short time-scale, might be limited.

We conclude that future temperature rise could have a negative effect on the nectar and flower production of Mediterranean plant species, particularly on the late-flowering species blooming from late spring to summer. The effect of climate warming on plant species and plant–pollinator interactions could be markedly different between seasons and these differences need be taken into account when estimating the overall effects of climate change. Having a more thorough knowledge of the effect of temperature rise on different plant traits, various species and the differences through seasons is essential to comprehend the effect of warming on whole communities and ecosystems through altered interaction networks. Our results on the effect of temperature

on the nectar secretion of different plants give a good basis for further studies on (i) the effect of different climatic factors (such as precipitation changes), (ii) effects on more detailed plant–pollinator interaction networks, and (iii) for tests under natural conditions, which could further advance our knowledge of the impact of climate change on ecosystem functioning.

AUTHOR CONTRIBUTIONS

TP conceived the idea and found funding for the study, and devised the experiments with the contribution by TT. KT conducted the experiments and analyzed the data. All authors contributed to the writing of the manuscript, which was led by KT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00874/full#supplementary-material

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