DUCKWEED: BIOLOGICAL CHEMISTRY AND APPLICATIONS

EDITED BY: Marvin Edelman, Klaus J. Appenroth and K. Sowjanya Sree
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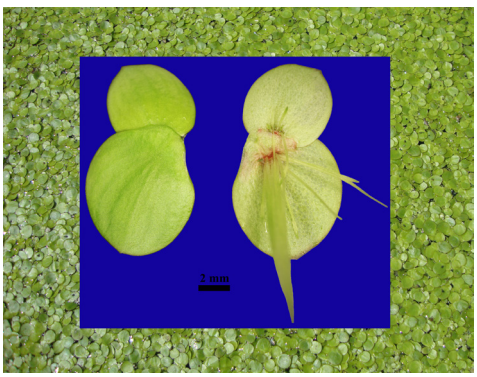
DUCKWEED: BIOLOGICAL CHEMISTRY AND APPLICATIONS

Topic Editors:
Marvin Edelman, Weizmann Institute of Science, Israel
Klaus J. Appenroth, Friedrich Schiller University Jena, Germany
K. Sowjanya Sree, Central University of Kerala, India



A pond surface covered by *Spirodela polyrhiza* (Greater Duckweed). Inset: Micrographs of the dorsal (Left) and ventral (Right) side of a colony of *S. polyrhiza.* (Credit: Dr. K. Sowjanya Sree, Central University of Kerala, Periye, India)

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EDITORIAL
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Editorial: Duckweed: Biological Chemistry and Applications

Marvin Edelman[1]*, *Klaus J. Appenroth*[2]* and *K. Sowjanya Sree*[3]*

[1] Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel, [2] Matthias Schleiden Institute – Plant Physiology, Friedrich Schiller University of Jena, Jena, Germany, [3] Department of Environmental Science, Central University of Kerala, Kasaragod, India

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Editorial on the Research Topic

Duckweed: Biological Chemistry and Applications

The duckweeds (Lemnaceae) are a family of simple, higher plants at the far end of the monocotyledon subdivision. All of the 5 genera and the currently accepted 36 species (Bog et al., 2019, 2020) are aquatic, fresh water plants, mostly floaters or slightly submerged, and all have extremely reduced anatomies. The leaf-like frond of the largest (*Spirodela polyrhiza*, Giant Duckweed) is only 1–1.5 cm in size, while the smallest (*Wolffia angusta*), which also represents the smallest Angiosperm, measures less than 1 mm. Duckweeds can flower, but normally propagate vegetatively, both in nature and in the laboratory, by budding from one or two meristematic zones within pockets in the frond. Species of the genera *Spirodela*, *Landoltia,* and *Lemna* have different numbers of adventitious roots with root caps, which may be more for stability in the water than nutrient uptake; the latter task is managed by the entire underside of the frond (Cedergreen and Madsen, 2002). Species of the genera *Wolffiella* and *Wolffia* are devoid of roots altogether.

As its name implies, duckweeds are a favorite food source for fowl, several fish and other animals as well (Appenroth et al., 2015). Under optimal conditions in nature or in the laboratory, several duckweed species can double their biomass almost daily representing the fastest growing Angiosperms (Sree et al., 2015). Depending on the cultivation conditions, the protein content of the biomass may reach up to 40% or more of the dry weight or the biomass may accumulate starch up to 50 % of the dry weight. In controlled conditions, they can be grown axenically, either autotrophically, mixotrophically, or even heterotrophically (Landolt and Kandeler, 1987). In addition, the genomes of some duckweeds (e.g., *S. polyrhiza*; genome size, 160 ± 3 Mbp/1C) are among the smallest for a higher plant (Wang et al., 2011; Bog et al., 2015). Presently, the genome sequence of *S. polyrhiza* clone 9509 represents the "gold standard" for duckweed genomes (Hoang et al., 2018). Coupled with the increasing abilities of several groups to genetically transform (Vunsh et al., 2007) various species of this aquatic family (recently, by CRISPR-Cas, Liu et al., 2019), one can think of "upcoming model system" or "biotech applications."

The present Research Topic demonstrates that research and practical application of duckweeds is flourishing, drawing the attention of researchers as well as application specialists from across the globe. This Research Topic contains a review article about genomes and transcriptomes of duckweeds by An et al. and 12 original research articles organized into separate sections devoted to "Ecology and Biomonitoring," "Biochemistry and Physiology," and "Biotechnology."

In the section "Ecology and Biomonitoring," Paolacci et al. report about competition between two duckweed species, *Lemna minuta* and *L. minor* in Ireland. Yang et al. test the effect of heavy metal Hg^{2+} on three species, *L. minor*, *L. gibba*, and *S. polyrhiza* and investigate the criteria to use duckweed species either for biomonitoring or phytoremediation. Gilbert et al. show that the association between bacterial endophytes and duckweeds belonging to a particular genus correlates with the indole related compounds produced by those endophytes.

Edited and reviewed by:
Jitendra Paul Khurana, University of Delhi, India

***Correspondence:**
Marvin Edelman
marvin.edelman@weizmann.ac.il
Klaus J. Appenroth
Klaus.Appenroth@uni-jena.de
K. Sowjanya Sree
ksowsree9@cukerala.ac.in

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In the following section "Biochemistry and Physiology," Appenroth et al. analyse both qualitatively and quantitatively the protein, fat, minerals, vitamins, and phytosterols, required for human nutrition, from all 11 species of *Wolffia*. Chakrabarti et al. report the profiles of amino- and fatty- acids in *L. minor* under non-sterile, mass production conditions, although the fertilizers used exclude use of the biomass for human nutrition. Utami et al. examine the effect of eight organic compounds including common phytohormones on *L. minor* and show that only ascorbic acid significantly stimulates biomass production. The cuticle on the abaxial and adaxial sides of duckweed is investigated by Borisjuk et al. who describe cuticle structure in relation to its function in interacting with different environments, like sunlight/air and nutrients/water. Evans et al. exploit the fact that duckweed can be cultivated under autotrophic, heterotrophic and mixotrophic conditions to apply N^{15}-labeled nitrate and ammonium under these conditions and investigate the size and dynamics of amino acid pools. Pagliuso et al. investigate all five duckweed genera concerning relative growth rates and content of soluble and cell wall carbohydrates suggesting apiose level as a biomarker for growth capacity of duckweeds.

In the final section, "Biotechnology," Heenatigala et al. outline stable transformation of *Wolffia globosa* for the first time using an *Agrobacterium tumefaciens* system, while Khvatkov et al. demonstrate stable transformation of *Wolffia arrhiza* to produce human granulocyte colony-stimulating factor (hG-CSF). Firsov et al. close the section with the expression of M2e from avian influenza virus H5N1 in transformed *L. minor* plants as a step in the development of broad-range oral vaccine against avian influenza.

AUTHOR CONTRIBUTIONS

All authors listed have drafted and revised the manuscript, and have made a substantial, direct and intellectual contribution to the work. Further, all the authors have approved it for publication.

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REVIEW
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Genomes and Transcriptomes of Duckweeds

Dong An[1], *Changsheng Li*[2], *Yong Zhou*[2], *Yongrui Wu*[2] *and Wenqin Wang*[1]*

[1] *Department of Plant Sciences, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China,* [2] *National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China*

Duckweeds (*Lemnaceae* family) are the smallest flowering plants that adapt to the aquatic environment. They are regarded as the promising sustainable feedstock with the characteristics of high starch storage, fast propagation, and global distribution. The duckweed genome size varies 13-fold ranging from 150 Mb in *Spirodela polyrhiza* to 1,881 Mb in *Wolffia arrhiza*. With the development of sequencing technology and bioinformatics, five duckweed genomes from *Spirodela* and *Lemna* genera are sequenced and assembled. The genome annotations discover that they share similar protein orthologs, whereas the repeat contents could mainly explain the genome size difference. The gene families responsible for cell growth and expansion, lignin biosynthesis, and flowering are greatly contracted. However, the gene family of glutamate synthase has experienced expansion, indicating their significance in ammonia assimilation and nitrogen transport. The transcriptome is comprehensively sequenced for the genera of *Spirodela*, *Landoltia*, and *Lemna*, including various treatments such as abscisic acid, radiation, heavy metal, and starvation. The analysis of the underlying molecular mechanism and the regulatory network would accelerate their applications in the fields of bioenergy and phytoremediation. The comparative genomics has shown that duckweed genomes contain relatively low gene numbers and more contracted gene families, which may be in parallel with their highly reduced morphology with a simple leaf and primary roots. Still, we are waiting for the advancement of the long read sequencing technology to resolve the complex genomes and transcriptomes for unsequenced *Wolffiella* and *Wolffia* due to the large genome sizes and the similarity in their polyploidy.

Keywords: duckweeds, genome size, genome, transcriptome, gene family

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Klaus J. Appenroth, Friedrich-Schiller-Universität-Jena, Germany

Reviewed by:
Alex Harkess, Donald Danforth Plant Science Center, United States
Evan Clarke Ernst, Cold Spring Harbor Laboratory, United States

***Correspondence:**
Wenqin Wang
wang2015@sjtu.edu.cn

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INTRODUCTION

The *Lemnaceae* family, commonly known as duckweeds, comprises five genera of *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia* within the monocot order of Alismatales. Each duckweed species presents their unique features. The *Spirodela* species are served as a promising aquatic reference genome due to its small genome size. The *Landoltia* and *Lemna* species are explored for applications in phytoremediation especitally in waste water treatment. The *Wolffiella*, and *Wolffia* are becoming renewable biorefinery feedstock given their fast growth and high starch content (Cheng and Stomp, 2009). Most duckweeds reproduce next generations by vegetative budding during spring and summer, while they become natural starch repository when they switch into

a dormant stage during winter time (Landolt, 1986). They are tolerant to various stresses, such as heavy metal, irradiations, and NH_4^+, serving an ideal system to study the response to abiotic stresses and providing an efficient way to restore the environment (Wang W. et al., 2016; Van Hoeck et al., 2017; Xu et al., 2018). Duckweeds can be co-cultured with municipal or swine wastewater to remove excess nitrogen and phosphorus, while the biomass can be readily converted into ethanol (Cheng and Stomp, 2009). Their unique characteristics and the economic potential have attracted a broad interest including (i) fast biomass accumulation, (ii) no competition with arable land, (iii) phytoremediation of wastewater or heavy metal polluted water, (iv) biofactories for pharmaceutical drugs (Stomp and El-Gewely, 2005), and (v) high starch content. To further release and improve the potential capabilities, it is becoming critical to interpreting the genome sequence, structure and gene functions. The falling cost of next generation sequencing (NGS) has made it accessible to an individual laboratory (Alkan et al., 2011). Still, sequencing a genome is a non-trivial work. It remains a challenging task within the reach of non-experts in terms of obtaining a high-quality assembly and annotation. The NGS reads are too short to resolve the high repetitive elements and polyploidy known in plant genomes, leading to incomplete or ambiguous assemblies (Li et al., 2018). Thus, the choice of plant genomes for sequencing has been driven mainly by cost efficiency and the avoidance of complexity. Thus, the species of *Spirodela* and *Lemna* with smaller genome size and less complexity have undergone genome sequencing (Wang et al., 2014a; Van Hoeck et al., 2015). The larger genomes of *Wolffiella* and *Wolffia* are not sequenced yet.

The alternative way is to only sequence RNA, the transcribed region from DNA template, which is the most efficient approach to get the essential information from the genome (Roberts et al., 2011; Trapnell et al., 2012). Transcriptome analysis of duckweeds is a great resource to understand how duckweeds get adapted to aquatic environments and seasonal changes, and how they respond to abiotic stresses. The invaluable resource will set the framework and stimulate new insights into discovering duckweeds' potential. In this review, we primarily introduce the sequencing strategy, the feature of genome and transcriptome along with the unique biology and physiology for the genera of *Spirodela*, *Landoltia*, and *Lemna*. The milestone of duckweed genome and transcriptome sequencing is summarized in **Figure 1**. We also give readers the future perspectives on sequencing the complex genome and transcriptome of duckweeds by using long read sequencing and the applications of the sequenced genomes in duckweeds research.

GENOME SIZING

With the fast development of sequencing technology especially NGS, the falling cost makes genome sequencing accessible to an individual laboratory. Still, genome assembly is a challenging task with respect to the inherent repeat content in DNA, the read length, and the sequencing depth. The cost-effective way is to choose the smallest genome that contains fewer repeats and is sequenced with a deeper coverage. The comprehensive study of genome sizing 23 duckweed species across 115 accessions were conducted by flow cytometry (FCM) before the initiation of duckweed genome sequencing. The ranges of genome size for each genus were summarized, presenting the big picture of genome variation in the duckweed family. It was found that the duckweed genome sizes varied 13-fold, ranging from 150 Mb in *Spirodela polyrhiza* to 1,881 Mb in *Wolffia arrhiza*. The 158-Mb genome of *Spirodela polyrhiza* 7498 was selected for sequencing. Surprisingly, the genome sizes of duckweeds correlated negatively with plant leaf (frond) size. The genus of *Landoltia* had a relatively stable genome size of ∼380 Mb, while *Lemna* showed a significant intraspecific and interspecific variation from 323 to 760 Mb, indicating polyploidy might be a major mechanism for the genome change (Soltis et al., 2015; Segraves, 2017; Van de Peer et al., 2017). The *Wolffiella* and *Wolffia* had the biggest genomes of 973 and 1,881 Mb, respectively (Wang et al., 2011). Given the small genome sizes of *Spirodela* (Wang et al., 2014a; Bog et al., 2015; Michael et al., 2017) and *Lemna* (Van Hoeck et al., 2015), they were selected for the first round of genome sequencing. The broad range of genome sizes makes duckweeds an invaluable system to study polyploidization and genome evolution.

The chromosome numbers reported for duckweeds were from $2n = 20$ to 126 with high variability (Landolt, 1986). Compared with Arabidopsis (5 chromosomes with a genome size of 157 Mb) (Bennett et al., 2003) and rice (12 chromosomes with a genome size of 466 Mb) (Yu et al., 2002), the individual chromosome size of *Spirodela polyrhiza* is small due to 20 chromosomes with 158 Mb, whereas it was reported that there was no obvious correlation between chromosome number and genome size (Hoang and Schubert, 2017).

The epigenetic modifications in duckweed chromatin played a significant role in gene transcription and translation. The studies of histone methylation and DNA modification including heterochromatic 5-mC, H3K9me2 and H3K27me1 in interphase nuclei found that the duckweeds with the genome size range of 158 to 1,881 Mb showed dispersed distribution of heterochromatin signatures. The immunolabelling pattern was similar to the early developmental stages of Arabidopsis nuclei, implying the association with the rapid growth of duckweeds but less dependent on the DNA content (Cao et al., 2015).

SPIRODELA GENOME AND TRANSCRIPTOME

The morphology of *Spirodela* is very simple with only leaves and roots. The genus has two species of *Spirodela polyrhiza* and *Spirodela intermedia* but multiple ecotypes (Les et al., 2002; Bog et al., 2015). The genome sequencing project indicated that it was subjected two ancient rounds of Alismatales-specific whole genome duplications (WGDs) and likely eliminated non-essential protein-coding genes, rDNA, and repeat elements to maintain its small genome size (Michael et al., 2017).

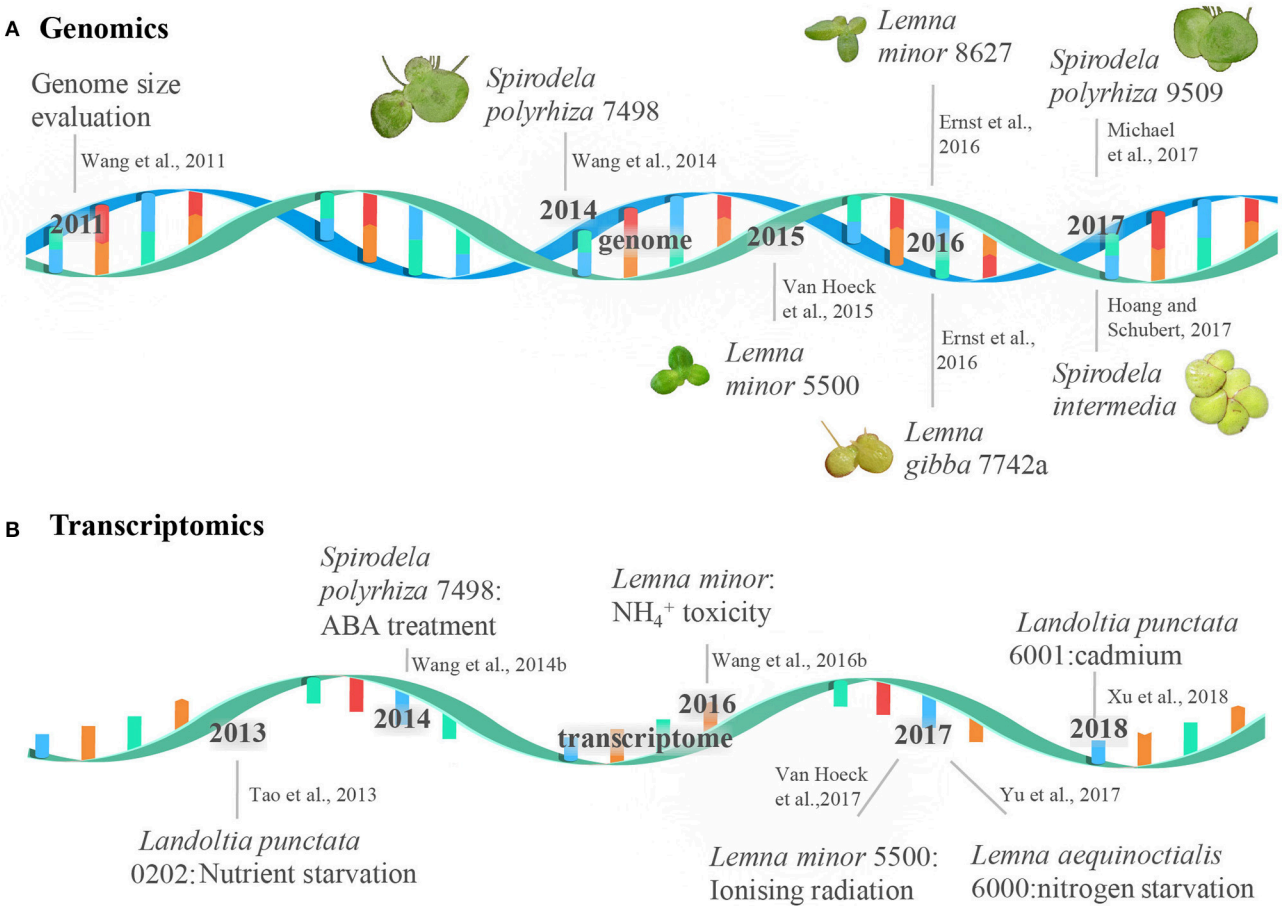

FIGURE 1 | The milestone of duckweed genome and transcriptome sequencing. The events of the genome and transcriptome sequencing were shown in a double-stranded DNA molecule **(A)** and a single-stranded RNA molecule **(B)**, respectively.

Genomes of *Spirodela Polyrhiza*

With the confirmation of the smallest genome of 158 Mb in the duckweed family and its basal ancestral phylogenetic position among duckweeds, *Spirodela polyrhiza* 7498 (Sp7498) was selected to whole genome sequencing as a basal monocot reference (Wang et al., 2011). With the prosperous development of the high-throughput DNA sequencing technology, the platform of 454 Life Sciences based on the "sequencing by synthesis" principle was used to sequence the genome of Sp7498, producing 21X coverage with 400–500 bp read lengths. BAC-end sequencing (BACs: Bacterial Artificial Chromosomes) from 15,260 clones with 100 Kb insertions was conducted by Sanger technology (**Table 1**). A complete physical map was developed by fingerprinting the BAC library with 10X coverage, providing an essential framework to order and join contigs assembled from 454 reads and BAC-end sequences (Wang et al., 2014a). To investigate the genome-wide intraspecific variation in *Spirodela* populations, another ecotype of *Spirodela polyrhiza* 9509 (Sp9509) was recently sequenced with 95X Illumina short reads and high-throughput genome mapping technology (Michael et al., 2017).

The final genome assembly for Sp7498 obtained 32 pseudomolecules with a contig N50 of 18 Kb and an N50 scaffold of 3.8 Mb, leaving about 10% unresolved gaps that could be repeat elements which were challenging to be assembled due to the limitation of the short reads (**Table 1**). By multicolor fluorescence *in situ* hybridization (mcFISH) using 96 BACs as probes with little repetitive sequences, the originally assembled 32 pseudomolecules were assigned into 20 chromosomes with an average resolution of 0.89 Mb (Wang et al., 2014a). The genome of Sp9509 was *de novo* assembled into 774 scaffolds with a contig N50 of 19 kb and a scaffold N50 of 4.3 Mb. With the guide of the previous assembled Sp7498 genome, Sp9509 scaffolds were joined into 23 larger ones with an N50 length of 5.8 Mb. To further close gaps and validate the assembled accuracy, a genome-wide physical map using the BioNano Genomics Iris System was developed for Sp9509 genome, which was resolved into 20 chromosomes with a scaffold N50 of 7.6 Mb (**Table 1**). It was found that the 20 chromosomes of *Spirodela* could be originated from seven ancestral chromosome blocks with two rounds of WGDs 95 million years (Myr) ago (Cao et al., 2016). The chromosomally integrated genome

TABLE 1 | The parameters for sequencing, assembling and annotating duckweed genomes.

Species	Genome size (Mb)	Platform	Sequencing coverage	Assembly program	Scaffold #	Scaffold N50	Contig N50 (Kb)	Repeat (%)	Protein coding gene #	References
Spirodela polyrhiza 7498	158	454 and Sanger	21	Newbler	32	3.8 Mb	18	17	19,623	Wang et al., 2014a
Spirodela polyrhiza 9509	160	Illumina and BioNano	95	AllPathsLG and SSPACE	20	7.6 Mb	19	23.8	18,507	Michael et al., 2017
Lemna minor 5500	481	Illumina	120	SOAPdenovo2 and SSPACE	46,105	23.6 Kb	20.9	61.5	22,382	Van Hoeck et al., 2015
Lemna minor 8627	800	Illumina and PacBio	NA	NA	NA	NA	222	NA	NA	Ernst, 2016
Lemna gibba 7742a	450	Illumina	NA	NA	NA	520 Kb	53	NA	21,830	Ernst, 2016

has accelerated the study of karyotype evolution in duckweed species.

The genome alignment between Sp7498 and Sp9509 revealed conflicts and identified potential misassembled sites in each genome, indicating that more PCR validations or long reads spanning over the junctions were required. There were 96 high-confidence structure variations (SVs) with the range of 1,000 to 100,000 bp between the two BioNano genome maps. The estimation of 81 rDNA copies was found in Sp9509, extremely less than Arabidopsis (570) and maize (12,000). The Southern blot analysis of four different accessions of *S. polyrhiza* revealed that the copy number of the rDNA clusters was all <100 copies (Michael et al., 2017).

The identified repeat elements constituted of ∼17% of the Sp7498 genome, most of which were long terminal repeat (LTR)-retrotransposons (Wang et al., 2014a). There were 25.25% repeats with 271 full-length long terminal repeats (LTRs). Comparative analysis with other species of Brachypodium, rice, and sorghum, *Spirodela* showed low transposon similarity, indicating a large evolutionary distance between *Spirodela* and the other monocots. The study of genome-wide bisulfite-sequencing found that the extent of cytosine methylation was higher for transposable elements than gene regions. The overall DNA methylation level in Spirodela was estimated to be 9%, which was considered to be the lowest in the tested plants of *A. thaliana* (32%), rice (39%), *Setaria italica* (44%) and *B. distachyon* (54%) (Michael et al., 2017).

Generally the high copy number tandem repeats (TRs) exist in the centromeres of a genome (Melters et al., 2013). The Sp7498 genome was predicted to have a 138-bp centromere repeat-like sequence, whereas Sp9509 was found a 119-bp TR on 19 out of the 20 chromosomes that contained high DNA methylation levels (Michael et al., 2017). The distribution of the 119-bp centromere repeat across some of the Spirodela chromosomes suggested that they were holocentric. This result was consistent with a dispersed heterochromatin signal observed in cytological studies (Cao et al., 2015).

The bioinformatics analysis predicted that there were 59 conserved microRNAs (miRNAs) of 22 families and 25 novel miRNAs. The small RNA sequencing validated 29 *Spirodela*-specific miRNAs in the genome of Sp9509. The sequence-based annotation identified five and three loci for miRNA156 and miRNA159 in Sp9509, respectively (Michael et al., 2017). In contrast, the Sp7498 genome included 24 loci encoded for miRNA156 and one locus encoded for miRNA159 (Wang et al., 2014a).

Spirodela polyrhiza has a small genome that is similar to Arabidopsis, but it has 30% fewer protein-coding genes of 19,623 in Sp7498 and 18,507 in Sp9509. Although the *Spirodela* genome exhibited reduced gene content, it shared a number of 8,255 common gene families with other plant species of Arabidopsis, tomato, banana, and rice (Wang et al., 2014a). Examination of copy number variation in *Spirodela* could give us indications of its compact and reduced morphogenesis, aquatic suspension and suppression of juvenile-to-adult transition.

Lignin is a major component of secondary cell walls to support land plants' up-straight height. It was found that

TABLE 2 | The contracted, expanded and conserved gene families in *Spirodela* compared with Arabidopsis and rice.

Species	Contracted gene family				Expanded gene family		Conserved gene family			
	Lignin biosynthesis*	Cellulose biosynthesis gene		Expansin	Glutamate synthase genes	miRNA156	Starch biosynthesis genes			
		CSL	GT31				AGPase	Starch synthase	Starch branching	Starch debranching
Spirodela	70	21	21	14	11	32	4	5	3	4
Arabidopsis	63	29	33	36	2	10	6	5	2	4
rice	156	36	39	-	3	19	7	10	3	4

**Lignin biosynthesis involves 10 gene families. Here shows the total copy number of 10 gene families. CSL, Cellulose synthase-like genes; GT, Glycosyl transferase; AGPase, ADP-glucose pyrophosphorylase.*

duckweeds contained very little lignin and cellulose content possibly owing to their specific aquatic habitat (Blazey and Mcclure, 1968). There were 141 and 156 gene copy numbers for lignin biosynthesis in sorghum and rice, respectively, while the *Spirodela* had only 70 members, consistent with its floating habitat that requires little strength to hold up their weight (**Table 2**; Wang et al., 2014a). Cellulose biosynthesis is critical for all plant cell wall synthesis. The gene families of cellulose synthase-like genes (CSL) and glycosyl transferases (GT31) for cell wall biogenesis were contracted in *Spirodela* in comparison with Arabidopsis and rice (**Table 2**) that was consistent with the low cellulose content in *Spirodela* (Wang et al., 2014a). Consistent with Sp7498, the gene family of expansin in Sp9509 was reduced to 14 in comparison of 36 members from Arabidopsis.

The fast growth of duckweeds needs highly efficient absorption of nutrients, for example, glutamate synthase genes that are dedicated to ammonia assimilation for duckweed fast propagation. It was found that up to four-time copies of glutamate synthase genes in *Spirodela* compared to Arabidopsis and rice (**Table 2**; Wang et al., 2014a; Michael et al., 2017). Consistent with rare flowering phenotype, miRNA156 playing a negative role in flowering and suppressing the juvenile-to-adult transition was identified to increase its copy numbers in *Spirodela* (**Table 2**). However, the copy numbers of starch biosynthesis genes encoding for ADP-glucose pyrophosphorylase (AGPase), starch synthase, starch branching enzyme, and starch debranching enzyme remained constant in *Spirodela*, Arabidopsis and rice (**Table 2**), indicating their conserved functions in starch biosynthesis.

Another aquatic plant *Zostera marina,* which is closely relative to *S. polyrhiza*, contains a genome size of 202.3 Mb and encodes 20,450 protein-coding genes, which is comparable with Sp7498. However, compared to 17% repeat elements in Sp7498, repeat elements in *Z. marina* accounted for 63% of the assembled genome. The gene family analysis showed that both species had gained 600 but lost 2000 gene families. The gene families of terpenoid genes, carbohydrate sulfotransferases, sulfatases, and MADS-box transcription factors responsible for flowering were dramatically reduced, indicating structural and physiological adaptations to their lifestyles and consistent with previous results (Olsen et al., 2016).

Chromosome Reconstruction of *Spirodela Intermedia*

The species of *Spirodela intermedia* has a similar genome size with *Spirodela polyrhiza*, while the genome has not been sequenced. The homology and chromosome rearrangements were investigated in *Spirodela intermedia* compared with the reference of *Spirodela polyrhiza* by using mcFISH with the identical set of 96 BACs from 20 chromosome pairs (Cao et al., 2016; Hoang and Schubert, 2017). It was found that *S. intermedia* was reconstructed into 18 chromosome pairs, two less than *S. polyrhiza*. Ten chromosome pairs were proved to be conserved between the two species, while six new linkages were detected possibly due to the rearrangements of chromosome breakage and translocations in *S. intermedia*. The reconstruction of karyotype provides a basis to study chromosome evolution in the genus of *Spirodela* and to assist *S. intermedia* genome assembly in future.

Transcriptome of *Spirodela Polyrhiza* Treated With Abscisic Acid

The genome sequence of *S. polyrhiza* provides a reference to analyse a whole transcriptome shotgun sequencing, also called RNA sequencing (RNA-Seq). RNA-Seq is a common technique to quantify gene expressions during plant development or stress stimuli. It was known that *S. polyrhiza* could survive through the cold winter or other extreme stress conditions by producing dormant fronds (turions) that were abundant of starch content. The addition of the hormone of abscisic acid (ABA) into growth medium can lead to turion formation quickly and starch synthesis (Smart and Trewavas, 1983). To better understand the mechanism of starch accumulation, RNA-Seq was conducted for the developing turions treated with ABA and the differentially expressed genes (DEGs) were defined. The functional terms of seed dehydration, carbohydrate, secondary metabolism, and senescence were enriched in up-regulated DEGs, whereas the genes responsible for rapid growth and biomass accumulation and protein synthesis were down-regulated (**Table 3**; Wang et al., 2014b). The identification and functional annotation of DEGs set a framework to understand the regulation of starch synthesis and the mechanism of dormancy. Moreover, the candidate genes could be further validated and engineered for practical applications such as starch and ethanol production.

TABLE 3 | The summary of RNA-Seq studies in duckweeds.

Species	Treatment	Assembled contig #	Assembled contig N50	Enriched biological processes		SRA	Platform	References
				Up-regulated	Down-regulated			
Spirodela polyrhiza	ABA induced turion formation	NA	NA	Seed dehydration, carbohydrate and secondary metabolism, and senescence	Rapid growth and biomass accumulation, carbon fixation, and protein synthesis	PRJNA205940	ABI_SOLID	Wang et al., 2014b
Landoltia punctata	nutrient starvation[#]	74,797	1,928	Starch accumulation, flavonoid biosynthesis,some ion transporters	Photosynthesis and respiration	PRJNA185389	Illumina HiSeq2000	Tao et al., 2013
	nutrient starvation[#]	155,903	2,190	Flavonoid and anthocyanin biosynthesis	Lignin biosynthesis	PRJNA185389	Illumina HiSeq2000	Tao et al., 2017
	cadmium toxicity	NA	NA	DNA repair, ROS metabolism, vacuolar sequestration, and protein metabolism	Protein phosphorylation, cellulose biosynthetic process, and cell wall organization	PRJNA361433	Illumina HiSeq4000	Xu et al., 2018
Lemna minor	ionizing radiation[#]	NA	NA	Cell wall modification, floavonoid biosynthesis, oxidative stress	DNA repair and mitosis	NA	Illumina HiSeq2000	Van Hoeck et al., 2017
	NH_4^+ toxicity	71,094	988	ROS scavenging, programmed cell death (PCD), and ligin biosynthesis	Ribosome pathway	PRJNA302233	Illumina HiSeq2500	Wang W. et al., 2016
Lemna aequinoctialis 6000	nitrogen starvation[#]	72,105	1,233	Starch biosynthesis	Nitrate reductase, glutamine synthase, and glutamate synthase	PRJNA368628	Illumina HiSeq2000	Yu et al., 2017

[#]*Nutrient starvation means that the duckweeds were transferred from nutrient-rich solution to distilled water. Nitrogen starvation was the treatment of duckweeds without any nitrogen in the medium. Ionizing radiation includes gamma- and beta-radiation with the addition of* ^{137}Cs *and a* ^{90}Sr *source in the nutrient medium.*

LANDOLTIA TRANSCRIPTOME

Landoltia genus contains only one species named *Landoltia punctata*. It can be readily distinguished from other duckweed species by root number in spite that is used to be referred as *Spirodela oligorrhiza*. *Landoltia* has a typical number of 2–4 roots for each frond in comparison with one root for *Lemna* and more than five roots for *Spirodela*.

Transcriptome of *Landoltia Punctata* Treated With Nutrient Starvation

It was reported that *L. punctata* contained high starch, rich flavonoid but little lignin during nutrient starvation, showing their potential to be developed as a resource plant for biofuel fermentation and flavonoid extraction. The starch percentage in *L. punctata* treated with nutrient starvation can reach 45.4% of the dry weight. The activity of ADP-glucose pyrophosphorylase, the most important key enzyme involved in starch synthesis was increased from the initial 9.6 units to 14.7 units per mg of total protein (Tao et al., 2013). A comprehensive transcriptome study (RNA-Seq) was conducted by transferring *L. punctata* to distilled water lack of nutrients (**Table 3**). Without *Landoltia* genome reference, short RNA-Seq reads were *de novo* assembled in order to build the transcriptome reference. A number of 74,797 contigs more than 200 bp were obtained. The N50 length of these contigs was 1,928 bp and the maximum length was 16,562 bp. The BLASTX found that 51,968 had significant hits that matched 25,581 unique protein accessions (Tao et al., 2013).

The short reads were aligned back to the assembled 74,797 contigs to quantify the gene expression profiling under nutrient starvation. The results showed that the transcripts encoding for key enzymes involved in starch and flavonoid biosynthesis were up-regulated, while the transcripts for photosynthesis and the rate-limiting enzymes of lignification were down-regulated (Tao et al., 2013).

A further investigation focused on flavonoid content was performed growing *L. punctata* in different culture medium (Tao et al., 2017; **Table 3**). Metabolome analysis detected a flavonoid accumulation from the original 4.51 to 5.56% (dry weight) after growing in distilled water. Consistent with metabolome, transcriptome analyses proposed that a special phenylalanine metabolic flux lead to the high flavonoid but the low lignin content. The integration of transcriptome, proteome, and metabolome indicated that high biomass with low starch and stable flavonoid content was produced in the full nutrient medium, while the accumulation of starch and flavonoids were stimulated by nutrient starvation. The plant growth retardant of uniconazole inhibited flavonoid biosynthesis but increase starch accumulation (Tao et al., 2017).

Transcriptome *Landoltia Punctata* Treated With the Heavy Metal of Cadmium

Landoltia punctata is an efficient, green, and economic approach to remove heavy metals and other pollutions from the water. Cadmium (Cd) is a heavy metal that is detrimental to the environment and crops. The screening test on 200 duckweed clones showed that *L. punctata* 6001 exhibited Cd tolerance. To further explore the molecular mechanism underlying the resistance to the heavy metal, a high-throughput transcriptome analysis was carried out for the Cd-treated samples (**Table 3**). DEG clustering and enrichment analysis showed the biological processes of DNA repair, ROS metabolism, vascuolar sequestration, and protein metabolism played a crucial role in Cd response and detoxification. Furthermore, the carbohydrate metabolic flux tended to be modulated in response to Cd stress (Xu et al., 2018).

The transcriptome sequence could not give the complete picture of the whole genome in terms of intergenic regions, introns, and repeats that could not be transcribed. Still, it provides a comprehensive view of gene expression with enough sensitivity and accuracy at certain developmental stages and under specific conditions. So far, none of *Landoltia punctata* has been sequenced. Therefore, the transcriptome sequence is particularly invaluable to understand their response to abiotic stresses and to benefit the basic research and practical applications.

LEMNA GENOME AND TRANSCRIPTOME

Lemna is a duckweed genus that is adapted to a broad climate region and extensively used in labs. The species of *Lemna minor* and *Lemna gibba* are the model systems to understand fundamental plant research such as the circadian clock, flowering mechanism, and genetic transformation (Yamamoto et al., 2000; Cedergreen and Madsen, 2002; Miwa et al., 2006). *Lemna gibba* is an aquatic higher plant that is used to evaluate the toxicity of pesticides by the Environmental Protection Agency (EPA) due to the facts of direct assimilation of chemicals from a liquid medium and their rapid growth (Brain and Solomon, 2007).

Lemna Minor 5500 Genome

Given the invaluable potential of *L. minor* for the physiology research and biotechnological applications, the accession of 5500 (Lm5500) with 481 Mb genome was assembled and annotated (Van Hoeck et al., 2015). The paired-end sequencing of HiSeq library covered 90X of the genome, while the MiSeq library represented 30X. The bioinformatic pipeline generated a moderate assembly including 49,027 contigs (N50 contig size 20.9 Kb) and 46,105 scaffolds (N50 scaffold size 23.6 Kb). It was well-known that the short reads could hardly span long repetitive sequences in a typical plant genome. The lack of mate-pair libraries, fosmids or BAC clones with large insertions had led to higher reduced contiguity for Lm5500 than Sp7498 (Xu et al., 2018). It was revealed that the Lm5500 genome contained 62% repetitive sequences including 31.20% retrotransposons, 5.08% DNA transposons, 3.91% tandem repeats, and 21.27% of other unclassified repeats (Van Hoeck et al., 2015). In comparison with ∼17% repeats in 158 Mb *S. polyrhiza*, repetitive elements in 481 Mb *L. minor* could explain 94.5% of the genome size difference. The structural annotation showed the average gene length was 2,738 bp comprising of 1,332 bp CDS, 208 bp exon, and 209 bp intron. The mean exon number per gene was 4.8. A number of 22,382 protein-coding genes were predicted in *L. minor*, similar to 19,623 members of *S. polyrhiza* (**Table 1**;

Wang et al., 2014a). There were 66.2% of *Lemna* proteome shared with the *Spirodela*. The GO analysis revealed that the gene functions involved in environmental adaptation, biomass production, and response to abiotic stresses were enriched. For example, the genes encoded for glutamine synthetases (GSs) and glutamate synthases (GOGATs) were greatly expanded to 12 and 21 members in *L. minor,* compared with 7 and 11 ones in *S. polyrhiza*, respectively, indicating their potential in wastewater remediation and fast growth.

Lemna Minor 8627 Genome

Another genome project of 800 Mb *Lemna minor* 8627 (Lm8627) was done by Martienssen Lab. *Lemna minor* had the ability to remove nitrogen and phosphorus from swine lagoon water (Stomp and El-Gewely, 2005). It was also an efficient system for genetic transformation using agrobacterium-mediated gene transfer protocol (Yamamoto et al., 2000; Cedergreen and Madsen, 2002). Lm8627 had nearly two times of Lm5500 genome size (481 Mb) and more than five times of Sp7498 (158 Mb). It was proposed that the *Lemna* ancestor experienced at least one recent WGD after the split of *Lemna* and *Spirodela*. The different degree of gene removal from duplicated genome resulted in various *Lemna* species. It was also hypothesized that the mechanisms of large repeat expansion or very recent WGD could be possible. The more sequenced genomes will clarify the relationships and the evolution history (Van Hoeck et al., 2015). The draft genome of *L. minor* 8627 was highly fragmented with a contig N50 of 65 Kb over nearly 40,000 contigs. The addition of long reads from PacBio sequencing greatly improved the contig N50 to 222 Kb and highly reduced the contig number to fewer than 9,000 (**Table 1**; Appenroth et al., 2015; Ernst, 2016). The genomic data can be retrieved from the database (https://www.Lemna.org), while the annotation is in progress.

Lemna Gibba 7742a Genome

To expedite the biology and genetics study for aquatic plants, Evan Ernst from Martienssen Lab sequenced 450 Mb L. gibba 7742a (Lg7742a) genome by Illumina short reads. The preliminary assembly of Lg7742a displayed fragmentation due to the absence of a physical or genetic map with a contig N50 of 53 Kb and a scaffold N50 of 520 Kb. The most recent annotation found 21,830 protein-coding genes (**Table 1**; Ernst, 2016), close to Lg5500 and Sp7498. The peer-reviewed website (https://www.Lemna.org) created by Cold Spring Harbor Laboratory (CSHL) made the draft genome sequence of *Lemna gibba* accessible to the community before their publication. The analysis tools of Gbrowse and BLAST were also available online.

The present version of *Lemna* genome will be highly helpful for deciphering the functional genomics contributed to absorbing nutrition from waste water and boosting the biomass accumulation.

Transcriptome of *Lemna Minor* Treated With Radiations and NH_4^+

Plants are constantly exposed to various radiations. The injury of chlorophyll and the depletion starch synthesis were observed under radiation (Farooq et al., 2000). To provide a better understanding of environmental radiation exposure, the gene expression responses measured by RNA-Seq were evaluated under different dose of gamma- and beta-radiation for 7 days (**Table 3**). The functional analysis revealed that *L. minor* could tolerate the lower dose of radiation by triggering the cell wall modification and flavonoid biosynthesis. However, the gene expression involved in anti-oxidative defense systems and ATP production were up-regulated, while the regulations of DNA repair and mitosis were decreased at the high dose of radiation (Van Hoeck et al., 2017).

Different with other plants, *L. minor* could grow well even in the high NH_4^+ environment, whereas little knowledge of the tolerance mechanism was known. Thus, the comparative transcriptome of *L. minor* under high concentration of NH_4^+ was studied (**Table 3**; Wang W. et al., 2016). It was reported that the genes encoding ROS scavenging enzymes, such as superoxide dismutase and peroxidase were detected to be up-regulated by NH_4^+ treatment. The increased lignin biosynthesis might also contribute to the resistance of NH_4^+ toxicity.

Transcriptome of *Lemna Aequinoctialis* Treated With Nitrogen Starvation

Lemna aequinoctialis 6,000 was sampled from Hunan Province of China. It was found that the starch content after 9 days of nitrogen starvation was three times higher compared to the pre-treatment. To identify the genes responsible for starch accumulation, the transcriptome profile of *L. aequinoctialis* 6000 was examined using RNA-Seq (**Table 3**). The generated sequencing data (25 Gb) were *de novo* assembled into 72,105 unigenes with an average length of 1,233 bp. The genes involved in nitrogen metabolism exhibited the earliest responses to nitrogen stress, whereas the genes responsible for carbohydrate biosynthesis were regulated subsequently. The expression of genes encoding nitrate reductase, glutamine synthetase, and glutamate synthase were down-regulated under nitrogen starvation. Consistent with the change of starch content, the activity of AGPase was significantly increased. It was concluded that the increase of ADP-glucose and starch contents under nitrogen starvation was a consequence of increased output from the gluconeogenesis and TCA pathways (Yu et al., 2017). The identified genes would be promising candidates for further genetic improvement of starch production. The mechanisms of starch accumulation during nitrogen starvation provided a foundation for the improvement of bioethanol production (Yu et al., 2017).

ORGANELLAR GENOMES

The chloroplast genomes of *Spirodela polyrhiza* (Sp7498), *Wolffiella lingulata* (Wl7289), and *Wolffia Australiana* (Wa7733) were assembled by computationally selection filter with a copy number-sensitive algorithm from total DNA sequencing (Wang and Messing, 2011). The cpDNA was a circular molecule of 168,704–169,353 bp containing a pair of 31,683–31,930 bp

inverted repeat regions (IRs). Comparative analysis suggested that the chloroplast genome was conserved in gene number and organization with respect to the reference genome of *L. minor* (Mardanov et al., 2008). However, substantial variations including nucleotide substitution, deletions and insertions occurred in non-coding regions compared to the chloroplast genomes of grass family (Wang and Messing, 2011).

The first complete mitochondrial genome of duckweeds was *Spirodela polyrhiza* assembled from ∼100x coverage of raw reads. The 228,493-bp genome was annotated into 57 genes (35 protein-coding genes, 3 ribosomal RNAs, and 19 tRNAs) (Logan, 2006; Wang et al., 2012). Further sequence analysis showed that 4.1% of the mtDNA was originated from chloroplast DNA, but very few nuclear sequences were found in mitochondrial DNA. The phylogenetic tree suggested that Spirodela shared a common ancestor with other monocots, but there is no obvious synteny in mitochondrial genomes between Spirodela and rice (Wang et al., 2012).

FUTURE PERSPECTIVE

NGS facilitates the unprecedented development of duckweed genomics and transcriptomics (**Tables 1**, **3**). With the overview of all sequenced duckweed genomes, we found that genomes of *Spirodela polyrhiza* (Sp7498 and Sp9509 with ∼158 Mb), *Lemna minor* (Lm 5500 with 481 Mb and Lm 8627 with 800 Mb) and *Lemna gibba* (Lg 7742a with 450 Mb) have been assembled into the contig N50 of 18–222 Kb. The protein coding gene numbers were comparable, whereas the repeat contents increased with the genome sizes (**Table 1**). The other genomes for *Landoltia*, *Wolffiella* and *Wolffia* are not available yet due to the higher repeat content and larger genome size. Given the average length of 8 Kb of retrotransposons (Kumar and Bennetzen, 1999), the short reads from NGS cannot fully span over the repetitive regions, leading to fragmented assemblies (Phillippy, 2017). A superior way to resolve transposon repeats is to generate long reads that are enough to exceed transposon regions (Li et al., 2018). The third generation sequencing platforms including PacBio single-molecule real-time (SMRT) sequencing and Oxford Nanopore sequencing produce up to 20 and 200 Kb reads that are able to efficiently assist the complex genome assembly. For instances, the incorporation of additional PacBio single-molecule long reads with short reads significantly improved *L. minor* 8627's assembly, resulting in the contig N50 increased from 65 to 222 Kb (Ernst, 2016). It was reported in the abstracts of Plant & Animal Genome Conference XXVI in 2018 that 375 Mb *Wolffia Australiana* was sequenced by PacBio long reads and BioNano Genomics optical mapping. They resolved large contigs with ribosomal DNA and identified two tandem repeats with high copy number that could be from centromeres (https://pag.confex.com/pag/xxvi/meetingapp.cgi/Paper/28517). Oxford Nanopore Technology is another competitive long read third generation sequencing technologies. Using Nanopore sequencing, the 1.2 Gb *Solanum pennellii* genome was assembled into a contig N50 of 2.5 Mb (Schmidt et al., 2017). Without *de novo* assembly of RNA-Seq reads, the long read sequencing, called isoform sequencing can obtain the full RNA molecular sequencing that could define the complete gene structure including untranslated regions of 5′ and 3′ ends, introns, and exons (Wang B. et al., 2016). It is believed that the long read sequencing technology would be applied in duckweeds in near future that not only improves the genome assembly but also enhances our understanding of the complex transcriptome.

The duckweed genomics gives us an important scientific advance that could revolutionize many aspects of molecular biology and genetics. The multiple sequenced genomes become essential resources to study the comparative genomics that are aligned to find out the similarities and differences as well as the genome structure and function. The further characterization of genomes could reveal the dynamics of gene families, the activity of transposable elements and the patterns of genome duplications. Still, the future of genomics research would target to understand the structural and functional components embedded in genomes either by mutation or the genome editing technology of CRISPR/Cas9. It is well-known that genes and their products cooperate in a complex and interconnected network. The elucidation of the genetic pathways of how they contribute to cellular and organismal phenotypes would be critical. The long goal of genomics would be the development of the transgenic duckweeds that improve the agronomic traits or extensively apply in the industry in terms of bioenergy and phytoremediation.

AUTHOR CONTRIBUTIONS

DA and WW did the literature search and wrote the manuscript, CL drew Figure 1, DA, CL, YW, YZ, and WW gave critical suggestions and made a proofreading.

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Competition Between *Lemna minuta*, *Lemna minor,* and *Azolla filiculoides*. Growing Fast or Being Steadfast?

Simona Paolacci[1,2]*, *Marcel A. K. Jansen*[1,2] *and Simon Harrison*[1,2]

[1] Enterprise Center Distillery Field, School of Biological, Earth and Environmental Sciences, University College of Cork, Cork, Ireland, [2] Environmental Research Institute, University College of Cork, Cork, Ireland

A substantial number of Lemnaceae are invasive outside their natural distribution area. *Lemna minuta* is considered invasive in several European countries, where it can occur in the same habitat as invasive *Azolla filiculoides* and native *Lemna minor*. In this study the presence, abundance and growth rates of all three species were monitored across 24 natural ponds and in a series of mesocosms in order to explore the importance of species invasiveness and habitat invisibility. Field monitoring showed that the distribution of the three species of macrophytes is heterogeneous in space and time. However, the data show no association of nutrient or light levels with plant distribution. Indeed, using reciprocal transplanting experiments it was demonstrated that all species are able to grow in all ponds, even ponds where the species do not naturally occur. It is concluded that distribution of *L. minor*, *L. minuta*, and *A. filiculoides* is not limited by the prevailing physicochemical characteristics of the ponds during the summer period. Remarkably, in these experiments *A. filiculoides* displayed the highest RGR, and exerted a negative influence on growth rates and surface cover of *L. minor* and *L. minuta*. Despite such apparent invasiveness, *A. filiculoides* was relatively rare in the study area. Rather, the species most abundant was *L. minor* which has the lowest RGR under field conditions in summer. Therefore, this study shows that the invasiveness of the species during the summer months is not necessarily reflected in the actual distribution pattern in natural ponds. In fact, alien *L. minuta* and *A. filiculoides* are under-represented in the monitored area. It is concluded that the interaction of several factors, including growth under winter-conditions and/or dispersal after disturbances, is the major determinant of the abundance and heterogeneous distribution of *L. minor*, *L. minuta*, and *A. filiculoides* in the study area.

Keywords: Lemnaceae, Azolla, invasive species, competiton, distribution pattern

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Duilio Iamonico, Sapienza Università di Roma, Italy

***Correspondence:**
Simona Paolacci
spaolacci@ucc.ie

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INTRODUCTION

Biological invasions have been increasing over the past 50 years (Levine and D'Antonio, 2003) and these invasions are a source of concern because of their negative effects on native species, habitats and biodiversity (McGeoch et al., 2010). Alien aquatic plants can have a negative impact on ponds, streams, rivers and wetlands. The dense growth of some alien aquatic plants can reduce flora richness and structural diversity and cause alterations in ecosystem function (Zedler and Kercher, 2004). Invasions can also have serious economic implications, particularly if they affect food

production, shipping, water-extraction, fisheries, tourism, and/or recreation. Across all ecosystems, there are estimated to be more than 1,000 invasive alien species in Europe that have been shown to cause a substantial ecological or economic impact (Vilà et al., 2009).

Understanding the factors that promote the invasiveness of alien species is fundamental in order to prevent invasions and restore invaded habitats (Byers et al., 2002). The ability of plants to invade a habitat is called invasiveness while, the susceptibility of an environment to the colonization and establishment by species not currently part of the resident community, is called invasibility (Davis et al., 2005). A biological invasion depends on both the invasiveness of the alien species and the invasibility of the habitat (Alpert et al., 2000). The degree of invasibility of a habitat depends on many factors including the species richness and the strength of interactions between species (Case, 1990). Resource availability, disturbance and environmental stressors have also been demonstrated to have an impact on the invasibility of habitats (Davis et al., 2000). Among the traits that seem to be correlated with a high invasiveness of a species are a broad native distribution range (Goodwin et al., 1999), rapid population growth (Rejmánek and Richardson, 1996), ability to deal with stress and disturbance and rapid dispersal (Alpert et al., 2000). The competitive strength of an alien species, relative to native species, also impacts on the success of an alien invasion (Alpert et al., 2000).

A substantial number of Lemnaceae species do occur outside their natural distribution range, and are considered to be invasive. For example, in Sweden five different species of non-native Lemnaceae have been found (*Lemna aequinoctialis*, *L. minuta*, *L. turionifera*, *Spirodela intermedia*, and *Landoltia punctata*) (Ryman and Anderberg, 1999). *Landoltia punctata* is native to south-east Asia and Australia, but is an alien in parts of Europe (Hussner, 2012), and in the U.S.A. (Jacono, 2002). *Lemna valdiviana* is native in the Americas but has become invasive in parts of southern Europe (Iberite et al., 2011). *Lemna gibba* is native in Europe, Asia and North America (Hussner, 2012), but alien invasive in parts of southern Africa (Henderson, 2007). In Ireland, as well as much of Europe, *L. minuta* is an alien invasive species. *L. minuta* (Least Duckweed) is native to temperate regions of North and South America (Stace, 2010). This duckweed naturally occurs in a wide range of habitats, including mountainous regions, up to 4000 m of altitude, to temperate and tropical regions (Landolt, 1986). Invasive, alien *L. minuta* has been spreading in Europe for the last 40 years (Gassmann et al., 2006). It is widespread in Europe, including Germany (Hussner et al., 2010), Belgium (Halford et al., 2011), Poland (Wójciak and Urban, 2009), Hungary (Lukács et al., 2014), France (Jovet and Jovet-Ast, 1966), Italy (Conti et al., 2005), and Malta (Misfud, 2010). In England *L. minuta* is becoming more prevalent, since being discovered in 1977 (Bramley et al., 1995). *L. minuta* was first found in Ireland in Co. Cork in 1993. Since, it has been reported at 133 lowland sites and is now considered an established species (Lucey, 2003). Another invasive species (but not belonging to the Lemnaceae) that is frequently observed in the same aquatic habitat as *L. minuta* is the freshwater fern *Azolla filiculoides*. This species is originally from North and South America where it is widespread from Patagonia to Alaska, including the Caribbean Islands (Wagner, 1997). The species has been recorded in 19 European countries, and based on the perceived threat level, the European and Mediterranean Plant Protection Organization (EPPO) included it on the EPPO List of Invasive Alien Plants (Hussner, 2012). *A. filiculoides* was introduced in the British islands at the end of the nineteenth century as an ornamental plant (Simonsen, 1968), but it is currently widely spread across these islands (Preston and Jane, 2014). The water fern has been reported to cause severe problems by impeding navigation, water flow and angling and by causing fish kills and damage to wetland biodiversity (Janes, 1998).

L. minuta and *A. filiculoides* often co-occur with the native *L. minor* (Preston and Jane, 2014) and appear to compete for the same habitat (Dickinson and Miller, 1998; Ceschin et al., 2016). A comparative approach with native species has often been used in studies of invasive species (Daehler, 2003; Bossdorf et al., 2005; Funk, 2008). This approach consists of a comparison of alien and native species, and attempts to identify traits associated with invasiveness such as biomass allocation, growth rate, size and fitness. Comparative studies are particularly meaningful when comparing species that occupy the same ecological niche, and/or species that are closely related, as this facilitates the identification of differences that may be responsible for invasiveness (Mack, 1996). Similarly, a comparative analysis of invaded and non-invaded habitats can identify characteristics that determine the invisibility of habitats.

In this study, the abundance, growth-rate and distribution patterns of two alien freshwater plants (*Lemna minuta* and *Azolla filiculoides*) were compared with those of *L. minor*. Environmental parameters were compared between invaded and non-invaded habitats. Specifically, the hypothesis was tested that a combination of high growth rates and nutrient enrichment will facilitate invasion. The data will contribute to the understanding of colonization events, and ultimately inform aquatic management approaches.

MATERIALS AND METHODS

This study is comprised of three parts.

1-Presence and Abundance of Three Species of Floating Macrophytes in Natural Ponds

Area Investigated

The ponds investigated are situated along the north and south banks of the River Lee in south-west Ireland, 5 km west of Cork City (**Figure 1A**). The area includes a range of small, still- and slow-flowing water bodies. A total of 24 still ponds (indicated in **Figure 1A**, coordinates in Supplementary Table 1) were selected for further study. The ponds selected included water bodies with heterogeneous characteristics (e.g., different North-South aspect, canopy-cover, proximity to farms and/or houses). Most ponds are <100 m^2, and the depth generally varies between 50 and 150 cm. The bedrock in the area is composed of Devonian

FIGURE 1 | Map of the area monitored **(A)**. Numbers indicate the sites investigated. Insert shows the location within Ireland. In **(B)** the average monthly temperature during the time frame of this study is showed. Data were provided by the Irish Meteorological Service, and measured at Cork airport meteorological station (51°50′50″ N 8°29′10″ W). Figure adapted from Paolacci (2016).

sandstone, covered by Carboniferous limestone. The area is used for agricultural and recreational activities. In winter, the entire area is subjected to inundation. Occasionally, some of the water bodies dry out, completely, or partially in summer.

Monitoring Approach

Field monitoring started in November 2013 and lasted till June 2015. The monthly average temperature during the period of field monitoring is shown in **Figure 1B**. A total of 6 observations were made of macrophyte abundance (November 2013, April, June and November 2014, May and June 2015), one of shading (in June 2014) and two of water nutrient content (April and June 2014).

The presence and abundance of three species of floating macrophytes was quantified for each of 24 still- and slow-flowing water bodies in the study area. A 50 × 50 cm floating quadrat was used to estimate the percent cover of each species in each quadrat. Four random throws of quadrats were carried out in each water body and the mean of the 4 quadrats was calculated. It was assumed that the percent cover of the quadrats reflected the percent cover of the water body. The values estimated were translated into the following classes:

1 –absent
2 –present (1–25% of the surface of the water body covered)
3 –abundant (26–75% of the surface of the water body covered)
4 –dominant (76–100% of the surface of the water body covered)

The canopy produced by other plants (trees, bushes and reeds growing around and inside the ponds) on the surface of the whole water body was visually estimated. Each site was classified according to the following scale:

Pond not shaded (0% canopy)
Pond partially shaded (up to 25% of canopy)
Pond mostly shaded (25–75% of canopy)
Pond completely shaded (75–100% of canopy)

Total Oxidized Nitrogen (TON) and Total Phosphorus (TP) concentrations were quantified in each water body in early spring (April 2014) and in early summer (June 2014). The content of TP in the water was determined using the ascorbic acid method (Murphy and Riley, 1962), while the TON content was measured using a DR 2800 Spectrophotometer following the cadmium reduction method (Koroleff, 1972).

2-Growth-Rates of Three Species of Floating Macrophytes in Natural Ponds

Ponds were selected based on the most abundant free floating macrophyte, generating three categories of ponds. In three selected ponds *L. minuta* was more abundant than the other species investigated, while in a further three ponds *L. minor* was most abundant. In the final three ponds none of the three species was present. The experimental design would also have required

the inclusion of three ponds in which *A. filiculoides* was the abundant species, but at the time of this experiment none of the sites presented this characteristic. In each of the selected ponds four plastic, floating enclosures were placed. Each enclosure was divided into 7 circular sub-units (short 12-cm lengths of plastic piping of 10.5 cm diameter, perpendicular to the water surface) inside which we grew all possible combinations of the three species (the three species alone, *L. minuta* with *L. minor*, *L. minuta* with *A. filiculoides*, *L. minor* with *A. filiculoides* and the three species all together) (**Figure 2**). The four enclosures were bound together and tied with a rope to the edge of the water body so that they had a certain degree of freedom, but they could not be dragged too far by the current or the wind. A net was placed over the enclosures to prevent birds from accessing the sub-units and to prevent leaves and other wind-blown material from accruing. Growth was quantified by placing biomass of each of the three species in the appropriate 10.5 cm diameter sub-unit (30 mg of *L. minuta*, 50 mg of *L. minor,* and 80 mg of *A. filiculoides* starting biomass). Plants were then allowed to grow for a period of 4 weeks. At the end of the 4 week period the plant material was collected, the different species were separated, weighted and the RGR was calculated according to the formula of Connolly and Wayne (1996):

$$RGR = \ln (Yf/Yi)/t$$

Where Yi is the initial biomass or the initial number of fronds, Yf is the final biomass or final number of fronds, t is the time in days and ln is the natural logarithm.

The experiment started the 20th of May 2014 and it ended the 20th of June 2014. Over the 3 weeks of the experiment the average maximum temperature registered at the Cork meteorological station was 15°C, while the average minimum temperature was 8.6°C.

3-Temporal Changes in Growth Rate and Abundance of Three Floating Macrophytes in Mesocosms

The mesocosm experiment started in November 2013 and finished in November 2014, and was designed to identify dynamic differences in abundance and growth rate across the different seasons. Twenty-eight mesocosms were constructed by sinking plastic containers 31 cm deep into the ground. Containers had a diameter of 50 cm. Mesocosms were filled with rain water after construction. No top-up was required for the duration of the experiments. In each mesocosm, a small amount of sediment (750 g) was added as a source of nutrients. This sediment was gathered from the same pond from which all the three macrophyte species were collected (pond 16 in **Figure 1A**). Following addition of sediment, mesocosms were left plant-free for 4 days in order to allow the sediment to settle, and for some of the nutrients contained in the sediment to dissolve in the water. When the experiment started the concentration of soluble orthophosphate (SRP) in the water was 0.03 ± 0.001 mg/l and the concentration of nitrate was 4.1 ± 0.3 mg/l. These concentrations were similar to those observed in several water bodies along the river Lee where the three macrophyte species are naturally present.

Mesocosms contained either single macrophyte species, combinations of two species, or a mixture of three species. All plant material used for the experiments was collected from a pond within the study area (pond number 16 in **Figure 1A**). In November 2013, a total of 5 cm^2 of floating macrophytes, of all the possible combinations of the three species, was placed in the mesocosms. Each combination was replicated four times, with replicate mesocosms located at random within the experimental array, to avoid spatial confounding. The mesocosms were covered with wide-mesh netting to prevent birds interfering with the experiment. The relative area occupied by each species in each mesocosm was estimated every month with the method of the point intercept (Floyd and Anderson, 1987).

Additionally, in each of the mesocosms containing only one species a small sub-enclosure with a diameter of 14.3 cm was placed in order to monitor growth rates every month (**Figure 3**). Every month 50 mg of the same species as present in the rest

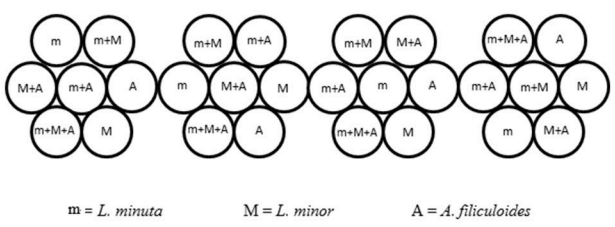

FIGURE 2 | Design of the floating enclosures for the field experiment. Four composite enclosures (each comprised of 7 sub-units) where placed in each of the 9 ponds selected for the experiment. The 4-times replicated enclosures contained all the possible combinations of the 3 species. Figure adapted from Paolacci (2016).

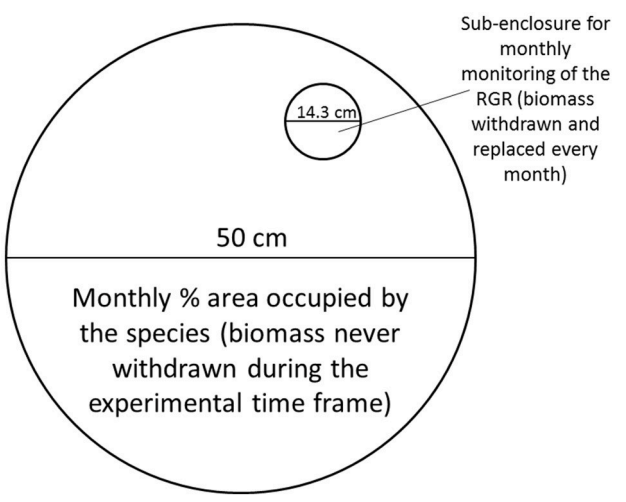

FIGURE 3 | Design of the mesocosm. The main compartment was used to monitor standing stock, while the sub-enclosure was used to determine growth rates for the three species when grown in allopatric conditions. Figure adapted from Paolacci (2016).

of the mesocosm was added to the sub-enclosure, and this was removed and weighed after 2 weeks. The RGR was calculated as detailed before. The monthly mean temperature for the experimental time frame can be observed in **Figure 1B**.

4-Data Analysis

All the statistic tests were performed using IBM SPSS Statistic 22.

Field Monitoring

The relation between the presence and/or abundance of the three macrophyte species and TON, TP and canopy cover was investigated by carrying out Kendall's Tau b test.

Field Experiment

A 2-way ANOVA was run in order to identify differences in RGR between the three species grown in allopatric conditions in the different categories of pond. For each of the three species, a 2-way ANOVA was run to analyse the differences in RGR for plants grown in different categories of ponds, and again to statistically compare growth in allopatric or sympatric conditions.

Mesocosm Experiment

A 2-way repeated measures ANOVA was used to analyse the differences in RGR and in percentage of surface cover between the three species, grown in allopatric conditions, in the different months of the year. For each of the three species, another 2-way repeated measures ANOVA was run in order to analyse the differences, in RGR or % of surface cover, between the species grown in allopatric or sympatric conditions. Sphericity was assessed with Mauchly's test. The degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. When a statistically significant interaction was found, an analysis of simple main effects was performed by running a one-way repeated measures ANOVA for the different subsets of variables. Bonferroni-corrected *t*-tests were applied for pairwise comparisons.

RESULTS

1-Monitoring the Presence and Abundance of Three Species of Floating Macrophytes in Natural Ponds

Of the three investigated species, *L. minor* and *L. minuta* were the most abundant in the 24 water bodies monitored during the 2 years of the study. *L. minor* was found in 12 water bodies, *L. minuta* in 11 water bodies and *A. filiculoides* only in three water bodies. In five water bodies, both *L. minuta and L. minor* were found, in three of them co-occurring at the same time, in the other two the two species were present at different times. In only one pond all three species were found together. In two other ponds *A. filiculoides* co-occurred with *L. minuta*. Thus, *A. filiculoides* never occurred in the absence of at least one *Lemna* species. No floating plants were observed in six of the 24 ponds (**Table 1**).

Analysis of water nutrient content revealed that Total Oxidized Nitrogen (TON) ranged between 0.01 and 5.207 mgl^{-1} (mean 1.798 mgl^{-1}) across all 24 water bodies in April 2014 and between 0.01 and 3.807 mgl^{-1} (mean 0.88 mgl^{-1}) in June 2014. Total Phosphorus (TP) ranged between 0.001 and 0.118 mgl^{-1} (mean 0.0132 mgl^{-1}) across the 24 water bodies in April 2014 and between 0.001 and 0.06 mgl^{-1} (mean 0.0062 mgl^{-1}) in June 2014.

There was no clear relationship between the percentage surface cover of the three species and TON and TP concentrations. Also the analysis of canopy cover did not reveal any significant correlation with the occurrence of the three species (**Table 2**).

In general, the three species were more widespread in the first year of monitoring. The comparison of June 2014 with June 2015 (months with the highest presence of the three species), showed that the number of ponds in which *L. minuta* was present decreased from 11 (in 2014) to 8 (in 2015). The 8 water bodies that contained *L. minuta* in 2015 also contained *L. minuta* in 2014. The number of sites in which *L. minor* was present decreased from 10 to 8. One of the ponds that contained *L. minor* in 2015 did not contain *L. minor* in 2014. The number of sites in which *A. filiculoides* was present decreased from 3 to 1.

2-Growth Rates of Floating Macrophytes in Ponds With Different Macrophyte Populations

Pond category (i.e., ponds naturally dominated by *L. minor*, or *L. minuta* or lacking macrophytes) did not affect the growth of the three species when these were introduced and raised in enclosures (**Figures 4A–C**). In fact, **Table 3** shows that for none of the three species the interaction between category of pond and species mix was significant. Yet, when *L. minuta* and *L. minor* were grown in the presence of *A. filiculoides* the RGR of the Lemnaceae was significantly reduced (**Figures 4A,B**). The RGR of *L. minuta* was significantly affected by both other species, Tukey *post-hoc* tests revealed that RGR of *L. minuta* grown alone was significantly greater than when grown with *L. minor* ($p = 0.002$), with *A. filiculoides* ($p < 0.001$) and with both species ($p < 0.001$). Also for *L. minor* RGR was significantly affected by the presence of the other species. Tukey *post-hoc* tests revealed that RGR of *L. minor* grown alone was significantly greater than when grown with *A. filiculoides* ($p = 0.003$) and with both species ($p < 0.001$).

3-Growth of Three Macrophyte Species Grown in Allopatric Conditions in Mesocosms, Over a 12 Month Period

Outdoor mesocosms were constructed to facilitate the study of growth throughout the four seasons. The analysis of growth under allopatric conditions showed that the three species had a reduced RGR in the colder months (from November to January), while their RGR increased from spring onwards. Growth of *L. minuta, L. minor*, and *A. filiculoides* peaked in the summer period between May and September. For *L. minuta* the highest RGR was 0.077 ± 0.015 day^{-1} in July. For *A. filiculoides* the highest RGR (0.12 ± 0.02 day^{-1}) was obtained in July. For *L. minor* RGR peaked in September (0.087 ± 0.007 day^{-1}). Lowest growth rates were measured in January, when none of the three

TABLE 1 | Presence and abundance of *L. minuta, L. minor*, and *A. filiculoides* in 24 ponds monitored at six different time points.

Site no.	TON (average April/June)	TP (average April/June)	Canopy shade in June	Species	Nov. 2013	Apr. 2014	Jun. 2014	Nov. 2014	May. 2015	Jun. 2015
1	0.01	0.005		*L. minuta*	•	●	●			
				L.minor						
				A.filiculoidesL.						
2	0.99	0.006		*L. minuta*						
				L.minor		▲	▲			
				A. filiculoidesL.						
3	2.507	0.004		*L. minuta*		•	•			
				L.minor					▲	
				A. filiculoides						
4	1.169	0.007		*L. minuta*						
				L.minor	▲	▲	▲	▲	▲	▲
				A. filiculoides						
5	1.252	0.002		*L. minuta*	•	●	●			•
				L.minor						
				A. filiculoides						
6	1.139	0.011		*L. minuta*		•	•			
				L.minor					▲	▲
				A. filiculoides						
7	0.01	0.06		*L. minuta*	•	•	●	•	•	●
				L.minor						
				A. filiculoides						
8	1.623	0.013		*L. minuta*	•	●	●		•	●
				L.minor						
				A. filiculoides	■	■	■		■	▲
9	2.566	0.019		*L. minuta*						
				L.minor		▲	▲		▲	▲
				A. filiculoides						
10	0.02	0.008		*L. minuta*						
				L.minor						
				A. filiculoides						
11	0.935	0.004		*L. minuta*						
				L.minor						
				A. filiculoides						
12	0.01	0.004		*L. minuta*		•	•	•	•	•
				L.minor						
				A. filiculoides						
13	0537	0.004		*L. minuta*						
				L.minor						
				A. filiculoides						
14	0.91	0.004		*L. minuta*						
				L.minor						
				A. filiculoides						
15	4.204	0.006		*L. minuta*						
				L.minor						
				A. filiculoides						
16	0.01	0.03		*L. minuta*	●	●	●		•	•
				L.minor	▲		▲			
				A. filiculoides			▲			

(Continued)

TABLE 1 | Continued

Site no.	TON (average April/June)	TP (average April/June)	Canopy shade in June	Species	Nov. 2013	Apr. 2014	Jun. 2014	Nov. 2014	May. 2015	Jun. 2015
17	2.95	0.002		*L. minuta*						
				L.minor	▲	▲	▲		▲	▲
				A. filiculoides						
18	0.01	0.001		*L. minuta*	●	●	●		•	•
				L.minor						
				A. filiculoides	■	■	■			
19	1.167	0.002		*L. minuta*	●	●	●	•	●	●
				L.minor		▲	▲		▲	▲
				A. filiculoides						
20	0.785	0.006		*L. minuta*						
				L.minor	▲	▲	▲	▲	▲	▲
				A. filiculoides						
21	3.336	0.005		*L. minuta*						
				L.minor	▲	▲	▲	▲	▲	▲
				A. filiculoides						
22	1.814	0.002		*L. minuta*						
				L.minor						
				A. filiculoides						
23	3.807	0.002		*L. minuta*	•	●	●	●	●	•
				L.minor						
				A. filiculoides						
24	0.38	0.002		*L. minuta*						
				L.minor			▲		▲	▲
				A. filiculoides						

L. minuta	*L. minor*	*A. filiculoides*	% Coverage
			Absent
•	▲	■	1-25%
●	▲	■	26-75%
●	▲	■	76-100%

Canopy
not shaded
partially shaded
mostly shaded
completely shaded

TON, Total Oxidized Oxygen; TP, Total Phosphorus, and canopy cover are also shown.

species grew. Both in December and February, only *L. minor* and *A. filiculoides* displayed growth, while *L. minuta* only displayed substantial growth from March (**Figure 5**).

Analysis using 2-way repeated measures ANOVA highlighted that there was a significant difference in RGR, both between species and between months. The interaction between the two factors was also significant (**Table 4**). In the colder months (from December from February) *A. filiculoides* did not significantly outgrow the other two species, but in March the RGR was higher than for *L. minuta* ($p = 0.042$) and *L. minor* ($p = 0.02$). The water fern continued to grow faster than *L. minuta* and *L. minor* in the following months until September (although not always significantly, see **Figure 5**).

4-Surface Cover of the Three Macrophyte Species Grown in Different Species Mixtures in Mesocosms

The percentage surface cover was measured every month for each of the three species grown in different mixtures. When grown alone, in the period December through to February, none of the species covered more than 2% of the surface area. From March to May only *L. minor* increased its percentage of surface cover. *A. filiculoides* increased its percentage of coverage only from May onwards, and *L. minuta* only from June. In the period July to November *A. filiculoides* covered up to the 100% of the mesocosm surface. The highest percent cover reached by *L. minuta* was 74.9

± 13.4% in August. For *L. minor,* the highest percentage of cover was 42.64 ± 9.17% in July (**Figure 6**).

A 2-way repeated measures ANOVA showed that there was a significant difference in surface cover between species and months. The interaction between the two factors was also significant (**Table 5**). From November to March 2015 there was no significant difference in surface cover between the three species, while, in April, *L. minor* surface cover was significantly higher than that by both *L. minuta* ($p = 0.024$) and *A. filiculoides* ($p = 0.046$). From June to November *A. filiculoides* always covered an area significantly greater than both *L. minuta* and *L. minor*. In these summer months, *L. minuta* covered an area greater than *L. minor*, but only in October was this difference significant ($p = 0.006$).

The monthly surface cover by each species grown alone was compared with the cover of that same species grown with one of the other two species or with both. Data analysis by 2-way repeated measures ANOVA was separately run for each species.

TABLE 2 | Correlation coefficient (Kendall's Tau *b*-test) between the percentage surface cover of *L. minuta*, *L. minor,* and *A. filiculoides* with Total Oxidized Nitrogen (TON), Total Phosphorus (TP) and Canopy cover.

	Correlation coefficient		
	L. minta	***L. minor***	***A. filiculoides***
TON	−0.193	0.044	−0.195
TP	−0.054	0.268	0.183
Canopy	−0.219	−0.174	−0.163

Correlation refers to the data collected in April and June 2014 for TON and TP and June 2014 for canopy cover. The data of percentage coverage by aquatic macrophytes refers to the same months of 2014.

Surface cover by floating macrophytes strongly depended on the season. For all the 3 species there was a significant interaction between species mixture and time (**Table 6**).

TABLE 3 | Summary of 2-way ANOVAs for each species, of the effects of mix of species (alone, with one of the other two species and with both) and pond category (ponds dominated by *L. minuta*, ponds dominated by *L. minor* or ponds with floating species absent) on RGR.

Source	Type III sum of squares	*df*	Mean square	*F*	Sig.
L. minuta					
Mix	0.023	3	0.008	165.625	0.000
Pond	0.000	2	0.000	2.221	0.130
Mix * pond	0.000	6	8.161E-05	1.729	0.157
Error	0.001	24	4.719E-05		
Total	0.133	36			
Corrected Total	0.025	35			
L. minor					
Mix	0.023	3	0.008	137.944	0.000
Pond	0.000	2	7.059E-05	1.244	0.306
Mix * pond	0.000	6	2.874E-05	0.507	0.797
Error	0.001	24	5.674E-05		
Total	0.179	36			
Corrected Total	0.025	35			
A. filiculoides					
Mix	0.001	3	0.000	3.579	0.029
Pond	0.000	2	8.412E-05	0.727	0.493
Mix * pond	0.001	6	0.000	0.867	0.533
Error	0.003	24	0.000		
Total	0.587	36			
Corrected Total	0.005	35			

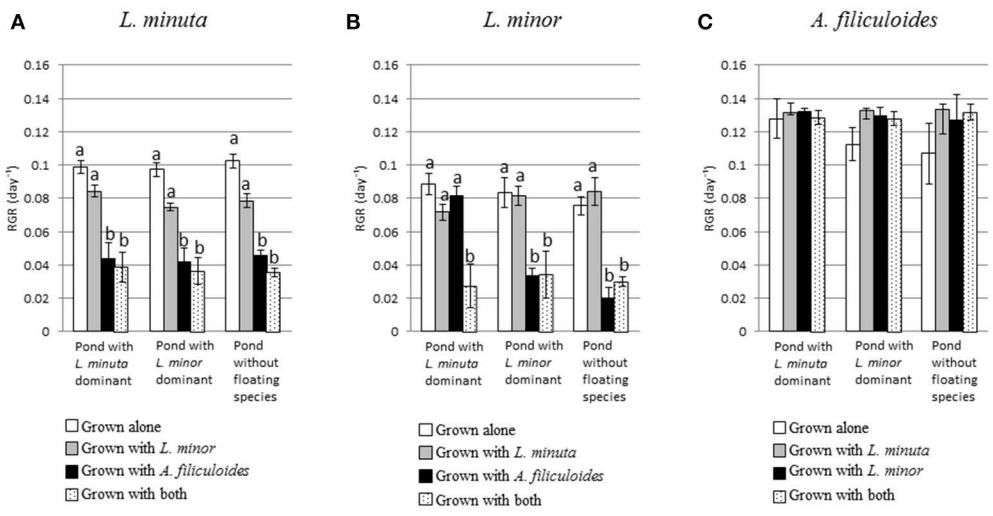

FIGURE 4 | Mean (±1 S.E.) RGR of *L. minuta* **(A)**, *L. minor* **(B)**, and *A. filiculoides* **(C)** grown in different mixtures of species (alone, with one of the other two species or with both) in the three different categories of ponds (ponds dominated by *L. minuta*, ponds dominated by *L. minor* or ponds with floating species absent). Different letters indicate significant differences between species for each pond type. Figure adapted from Paolacci (2016).

The surface area occupied by *L. minuta* was significantly affected by both the species mixture and the month of the year (**Table 6**). Surface cover was strongly reduced when this species was cultured in the presence of the other two species, but only from July to November (**Figure 6A**). In July, *L. minuta* surface cover was reduced when grown together with *L. minor* and surface cover was nearly completely suppressed when *A. filiculoides* was present in the culture mix. A similar situation was observed also in the following months.

The surface area occupied by *L. minor* was also significantly affected by the month of the year, while the effect of the species mixture was not statistically significant (**Table 6**). The percentage surface cover of this species was reduced by the presence of *A. filiculoides*, in the period from July to November, but not significantly. The presence of *L. m inuta* had no effect on *L. minor* surface area (**Figure 6B**).

The surface area occupied by *A. filiculoides* was significantly affected by both the species mixture and the month of the year (**Table 6**). The most evident difference between species mixtures occurred in May and June, when *A. filiculoides* surface cover was reduced by the presence of the other species. From July to November there was a smaller difference between the surface coverage for this species grown alone and in sympatric conditions (**Figure 6C**).

TABLE 4 | Results of 2-way repeated measures ANOVA.

	Source	Type III sum of squares	*df*	Mean square	*F*	Sig.
Species	Greenhouse-Geisser	0.030	1.282	0.023	22.444	0.009
Time	Greenhouse-Geisser	0.116	1.584	0.074	35.030	0.002
Species * time	Greenhouse-Geisser	0.036	2.290	0.016	6.868	0.021

Interaction between species (L. minuta, L. minor, and A. filiculoides) and time on RGR of the three species, grown in allopatric conditions in mesocosms.

DISCUSSION

The invasiveness of species and the invasibility of habitats are considered the key complementary parameters that determine the potential success of biological invasions (Alpert et al., 2000). Factors that increase habitat invasibility include high resource availability, limited competition with species present, ecological disturbances and the absence of environmental stressors (Alpert et al., 2000). Factors that increase the invasiveness of a species include rapid population growth, ability to deal with stressors and/or disturbances, and rapid dispersal. While invasiveness and invasibility have been investigated in depth separately, there is a gap in the literature for studies analyzing their antagonistic and/or synergistic effects on species distribution. This study integrates analysis of invasiveness and invisibility by measuring simultaneously growth rates, distribution and occurrence of two invasive (*L. minuta* and *A. filiculoides*) and a native species (*L. minor*) as well as resource availability and seasonality across a series of natural ponds and mesocosms.

Distribution of *L. minor*, *L. minuta*, and *A. filiculoides* Is Not Associated With Resource Availability

Surface cover was quantified for three free floating macrophytes across 24 natural ponds, which are all located in a wetland along the river Lee in south-west Ireland. Despite the apparent similarity of the ponds, as well as the proximity to one another, Lemnaceae (i.e., *L. minor* and/or *L. minuta*) were found in 18 ponds, but not in another 6 ponds, in the 2014 survey. Native *L. minor* was the most common species in ponds within the study area (12 out of 24), closely followed by the alien *L. minuta* (11 out of 24). The other alien, *A. filiculoides* occurred in only 3

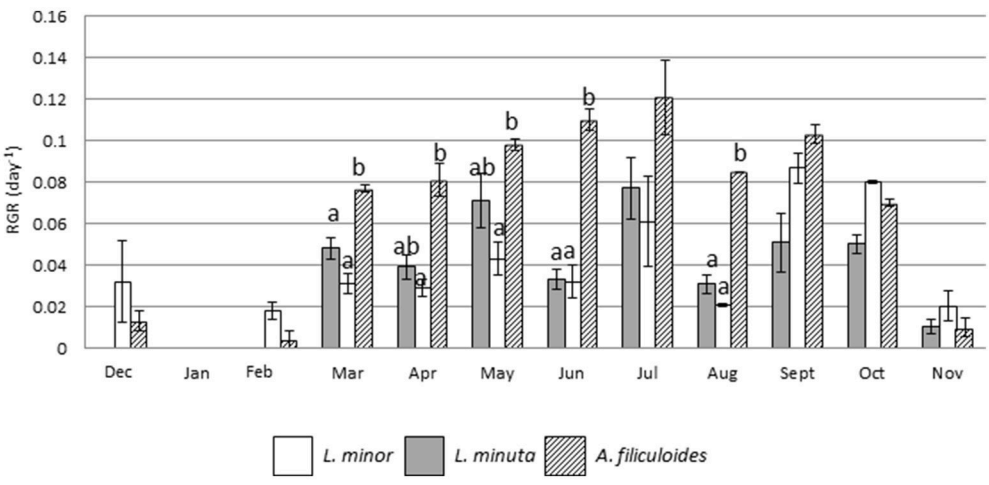

FIGURE 5 | Mean (±1 S.E.) RGR of *L. minuta*, *L. minor*, and *A. filiculoides*, grown in sub-enclosures in outdoor mesocosms from December 2013 to November 2014. Different letters indicate significant differences for each month.

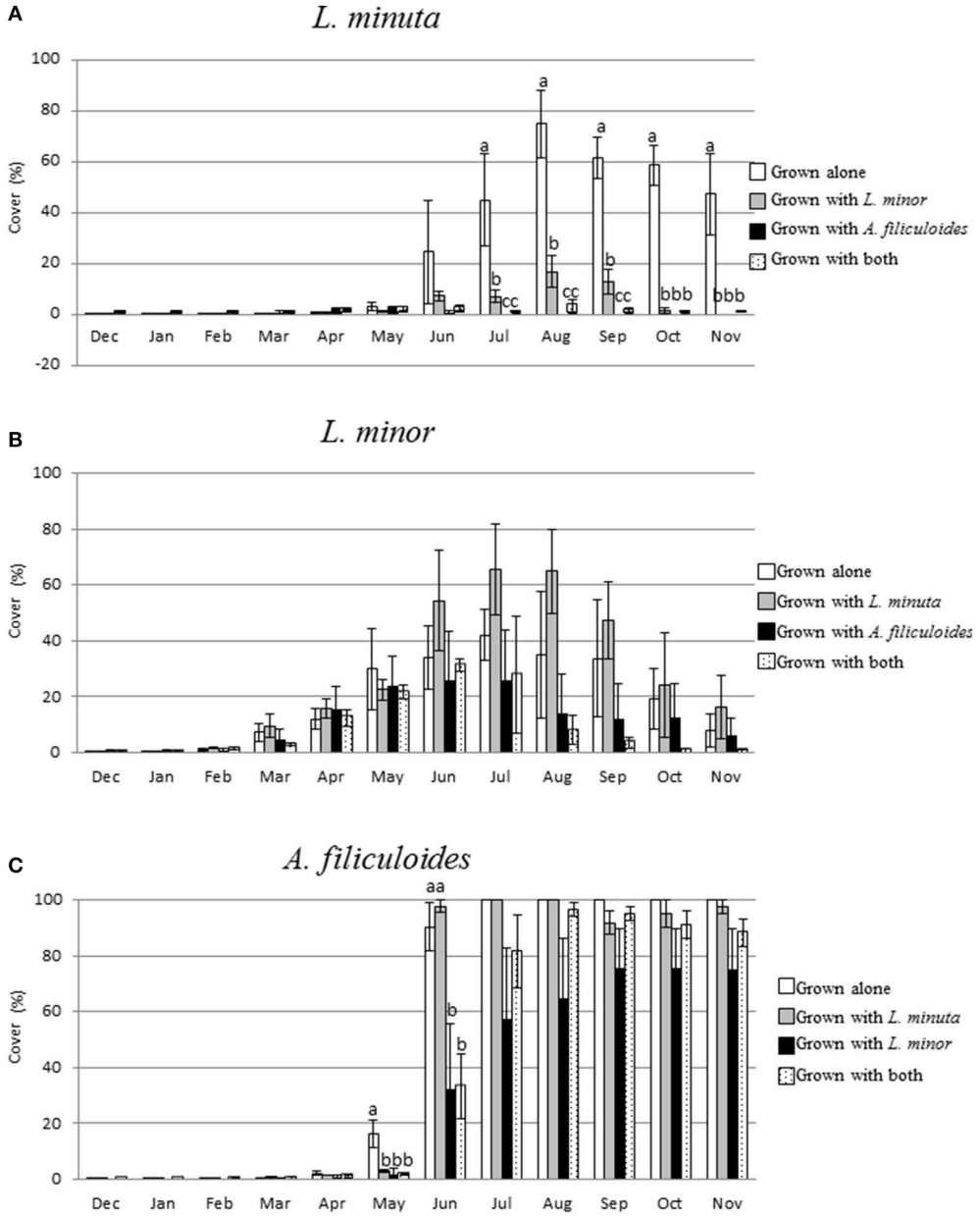

FIGURE 6 | Mean (±1 S.E.) Percent surface cover of *L. minuta* **(A)**, *L. minor* **(B)**, and *A. filiculoides* **(C)**, grown in different mixtures (alone, with one of the other two species or with both), in outdoor mesocosms from December 2013 through to November 2014. Different letters indicate significant differences between species in each month. The total surface area (100%) was 1,753 cm^2. Figure adapted from Paolacci (2016).

out of 24 ponds, and can thus be considered rare in the study area. Interestingly, the 2015 survey showed that some ponds that contained *L. minor* or *L. minuta* in 2014, lacked these species in 2015 (e.g., ponds 1 and 2). Other ponds that did not contain *L. minor* in 2014, did so in 2015 (e.g., pond 3 and 6). Thus, it can be concluded that the distribution of the three species of free floating macrophytes is heterogeneous in both space and time. Spotty distribution of Lemnaceae has been reported previously. McLay (1974) reported heterogeneous distribution of *L. perpusilla,* and that the presence of the species was negatively associated with exposure to wind and waves, and positively linked with the presence of *Potamogeton* and *Scirpus*. Similarly, Kline and McCune (1987) reported on the heterogeneous distribution of *Wolffia columbiana* and *Wollfia punctata* across a series of small potholes clustered together in a small area in Montana, USA. As reported in this study, a heterogeneous distribution of Lemnaceae was found, notwithstanding distances of <50 m between potholes. In the study by Kline and McCune (1987) it was concluded that the heterogeneous distribution of Lemnaceae reflected environmental parameters. Although the heterogeneous

TABLE 5 | Results of the 2-way repeated measures ANOVA.

Source		Type III sum of squares	*df*	Mean square	*F*	Sig.
Species	Greenhouse-Geisser	28118.260	1.069	26312.858	13.462	0.031
Time	Greenhouse-Geisser	115234.953	1.355	85037.560	55.056	0.001
Species * time	Greenhouse-Geisser	39591.563	1.700	23286.138	9.429	0.021

Interaction between L. minuta, L. minor, and A. filiculoides and time in the comparison of the surface cover by the three species grown in allopatric conditions.

TABLE 6 | Results of a 2-way repeated measures ANOVA.

Source		Type III sum of squares	*df*	Mean square	*F*	Sig.
L. minuta						
Mix	Greenhouse-Geisser	21169.910	1.231	17201.353	69.012	0.001
Time	Greenhouse-Geisser	11512.566	1.439	7999.795	17.413	0.010
Mix * time	Greenhouse-Geisser	24376.574	1.472	16560.542	10.882	0.022
L. minor						
Mix	Greenhouse-Geisser	9772.164	1.715	5697.963	1.346	0.331
Time	Greenhouse-Geisser	33933.470	1.414	24001.549	13.787	0.016
Mix * time	Greenhouse-Geisser	12316.381	1.868	6594.991	1.722	0.260
A. filiculoides						
Mix	Greenhouse-Geisser	10883.911	1.174	9269.019	5.053	0.096
Time	Greenhouse-Geisser	351738.347	1.603	219371.285	215.791	0.000
Mix * time	Greenhouse-Geisser	16870.522	2.185	7722.444	2.485	0.156

Interactions between different species mixtures and time in the comparison of the surface cover of the three species.

distribution in space is, thus, a more commonly reported phenomenon, the current study adds an extra layer of complexity by demonstrating a heterogeneous distribution in time.

In general, distribution of a species depends on the match of an array of physico-chemical parameters with the specific environmental requirements of the species (Santamaría, 2002). For example, a study by Peeters et al. (2016) revealed the relative importance of phosphorus for growth and competitiveness of free-floating macrophytes, and the presence of many species of aquatic macrophytes is associated with eutrophic conditions (Carbiener et al., 1990). The study presented here revealed a substantial gradient of light and nutrients across the 24 natural ponds in the study area. However, the data presented in this paper show no association of nutrient or light levels with distribution suggesting that physicochemical conditions in ponds fulfill minimal requirements for the three species. In support, previous experimental work has shown that both *L. minor* and *L. minuta* grow well on a broad range of nutrient conditions (Paolacci et al., 2016). Furthermore, on a global scale, aquatic species tend to be more widespread than closely related terrestrial species, and distribution patterns are typically less affected by environmental factors (Santamaría, 2002).

The explanation that all ponds fulfill the minimal requirements for growth of all three macrophyte species, triggers the challenging question why 6 out of 24 ponds have no free floating macrophyte cover in 2014. This is a particularly intriguing question as some of these ponds contained substantial amounts of floating macrophytes in 2015. Using reciprocal transplanting experiments (**Figure 4**), it was demonstrated that all species are able to grow in all ponds, even ponds were the species do not naturally occur. It might have been anticipated that actual growth rates (RGR) depend on light and nutrient levels. However, this was not the case. Indeed, in complex natural environments, effects of nutrients or light can be masked by other environmental factors, leading to the lack of correlation between resource availability, presence and growth rate. This is consistent with work by Makkay et al. (2008) who reported that in many cases single physical or chemical variables fail to explain the variation in aquatic plant community composition. A detailed study of the replacement of *L. minor* by *L. minuta* in central Italy, also led to the conclusion that environmental factors cannot explain the outcompeting of *L. minor* by *L. minuta* (Ceschin et al., 2016). Thus, based on the data presented in this paper it is concluded that distribution of *L. minor*, *L. minuta*, and *A. filiculoides* is not limited by the prevailing physicochemical characteristics of the studied water bodies during the summer period, and in the experimental area.

A High RGR Is Not Associated With High Abundance And/or Wide Distribution

Analysis of growth rates revealed that all species can grow in all ponds tested. Highest growth rates (RGR) in the field were noted for *A. filiculoides*. This species also had a dramatic negative effect on the RGR of the two species of Lemnaceae, when cultured together in close proximity. Analysis of the areas covered by the three macrophytes in the mesocosms further highlighted that

A. filiculoides was able to reduce the coverage of *L. minor* and *L. minuta*. In contrast, in the absence of *A. filiculoides* both *L. minuta* and *L. minor* can cover a substantial part of the available surface (**Figure 6**). *A. filiculoides* appeared to suppress the growth of the two *Lemna* species, possibly due to its higher growth rate as argued by Filizadeh (2002), or possibly due to its morphological features that facilitate it taking over the space available. The importance of morphology was recognized by Clatworthy and Harper (1962), who observed that the floating macrophyte *Salvinia minima* can outcompete *Spirodela polyrhiza* through physically overtopping the latter. Irrespective of the underlying mechanism of competition, we found that a relatively rare species (i.e., *A. filiculoides*) displays the highest RGR, the highest surface cover and is the most competitive, in the studied ponds, during the summer. Conversely, *L. minor* has the widest distribution in the ponds studied (**Table 1**), but displays the lowest RGR values (**Figure 4**) in monocultures under field conditions. In a way these data are surprising, as many, but not all, studies have associated high Relative Growth Rates (RGR) with competitiveness (Grotkopp and Rejmánek, 2007; Dawson et al., 2011). While "competitors" typically display a high potential RGR, stress adapted plants are characterized by a more modest RGR (Grime and Hunt, 1975). Interestingly, Santamaría (2002) has characterized aquatic habitats as inherently stressful, implying that high RGR values are not necessarily relevant to explain growth and competitiveness. Consistently, based on the data presented in this paper, it is concluded that the RGR, even when measured in a natural habitat, is not necessarily a good indicator for either abundance or distribution.

Differences in the Phenology of Three Free Floating Macrophytes

The period from late spring to early autumn, is the main period of growth for free floating aquatic macrophytes in Ireland (**Figure 5**). To explore in more depth the importance of seasonal growth, a mesocosm experiment was used to monitor RGR throughout the seasons. In general, RGR values tend to be highest in summer, and lowest in winter (**Figure 5**) patterns that are consistent with both lower temperatures and light-doses in the latter period. Percent surface cover was also lowest in winter (**Figure 6**). However, there are significant distinctions between the three species studied. *L. minor* displayed a significantly higher RGR than the other two species in winter (December–February) (**Figure 5**). *L. minor* also displayed significant surface cover in March and April, well ahead of the two other species (**Figure 6**). Phenological variations, whereby a species exploits resources at a time that other species are not active, can play an important role in competitive relationships (Regehr and Bazzaz, 1976). An "early start" can give a species a competitive advantage relative to a fast growing competitor that "arrives" later in the growing season. The relative ability of *L. minor* to grow in winter has been noted before. Reddy and DeBusk (1985) showed that the growth of *L. minor* is less influenced by seasonal changes than that of the water fern *A. caroliniana*. Also, Paolacci et al., (submitted) showed that *L. minor* can grow under lower temperatures than *L. minuta*, under laboratory conditions. Thus, we conclude that the three species of free floating macrophytes have different phenological cycles, with *L. minor* being able to maintain a low growth rate throughout much of the winter period in Ireland, and this may confer a competitive advantage.

What Determines the Heterogeneous Distribution of Three Free Floating Macrophyte Species Across the Study Area?

A remarkable finding of this study has been that the species with the lowest summer RGR, i.e., *L. minor*, is most widely distributed throughout the study area. In contrast, the species with the fastest summer growth, i.e., *A. filiculoides*, is relatively rare. Furthermore, this study has revealed a heterogeneity in time, with variations in the distribution of floating aquatic plants across ponds, between subsequent years. These two findings trigger the question what determines the distribution and abundance of the three species of free floating macrophytes in the studied system of ponds. Here we identify two important elements that determine this heterogeneous distribution.

(1) Dispersal and heterogeneous distribution

Shifts in the community composition of floating plants have been observed in response to seasonal environmental factors such as flooding, drought and extreme temperatures (Bornette and Puijalon, 2011; O'Farrell et al., 2011). Ponds in the area investigated are subject to flooding in winter, at which all ponds become connected and subject to substantial currents that may wash away free floating macrophytes. Ponds may also be subject to drought in summer, at which stage there may be no surviving free floating macrophytes. In this study it was concluded that all studied species can grow during the summer in all ponds. Therefore, it can be argued that re-colonization of these ponds after winter flooding and/or summer drought is a determinant of vegetation composition and that dispersal pathways need to be considered when analyzing vegetation dynamics. A study by Nishihiro et al. (2014) linked heterogeneous distribution of floating-leaved *Trapa japonica* to limitations in seed dispersal. Conversely, the "spotty" distribution of floating plants (Wolek, 1983; Kline and McCune, 1987) has sometimes been attributed to chance dispersal. However, bird mediated dispersal may facilitate targeted distribution in the waterfowl rich study area. Bird mediated dispersal has been well described for short distance dispersal (Jacobs, 1947; Reynolds et al., 2015), especially for species of Lemnaceae (Coughlan et al., 2015a,b). Thus, the possibility that Lemnaceae distribution patterns in the field study area reflect dispersal patterns needs to be considered.

(2) Phenological factors

In this study it was found that native *L. minor* displays stronger growth during the winter months than *L. minuta* and *A. filiculoides* (**Figures 5**, **6**). Potentially this can give *L. minor* a competitive advantage over the other species. Thus, it can be envisaged that a long winter and/or cool spring will benefit *L. minor,* and result in increased abundance. Conversely, a

warm spring and/or hot summer might favor the other two species. Such a scenario is in agreement with work by Dickinson and Miller (1998), who showed that the floating aquatic macrophyte *Salvinia minima* was highly competitive during the summer, negatively affecting cover by both *Azolla caroliniana* and *Spirodela punctata*. However, competitive superiority of *S. minima* was found to be seasonal, with summer gains being reversed due to a relative intolerance of winter conditions. Similarly, Peeters et al. (2013) observed that milder winters are correlated with a relatively higher abundance of free-floating plants and, as a result of shading, a reduced presence of submerged plants. Thus, the possibility that *L. minor* prevalence in the field study area reflects winter growth needs to be considered.

CONCLUSION

A. filiculoides displays the highest RGR in this study, and exerted a negative influence on growth rates and surface cover of *L. minor* and *L. minuta*. Despite such apparent invasiveness, *A. filiculoides* was relatively rare in the study area. Rather, the species most present throughout the study area was *L. minor* which has the lowest RGR under field conditions in summer. Therefore, this study proves, for the first time, that the invasiveness of the species during the summer months is not necessarily reflected in the actual distribution pattern in natural water bodies. In fact, the alien species *L. minuta* and *A. filiculoides* are under-represented in the monitored area. It is concluded that the interaction of several factors, including growth under winter-conditions and/or dispersal after disturbances, is the major determinant of the abundance and distribution of *L. minor*, *L. minuta* and *A. filiculoides* in the study area. These results can have implications in the management of invasive species, suggesting that an integrated analysis of invasiveness and invasibility is necessary to decide whether an intervention is required or not.

AUTHOR CONTRIBUTIONS

SP designed and carried out the experiment, analyzed and interpreted the results and wrote the paper. SH supervised the design and execution of experiments, the data analysis, the interpretation and the manuscript. MJ supervised the design and execution of experiments, the interpretation of results and 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/fchem.2018.00207/full#supplementary-material

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A Comparison of Growth on Mercuric Chloride for Three *Lemnaceae* Species Reveals Differences in Growth Dynamics That Effect Their Suitability for Use in Either Monitoring or Remediating Ecosystems Contaminated With Mercury

*Jingjing Yang[1], Gaojie Li[1], Anthony Bishopp[2], P. P. M. Heenatigala[1], Shiqi Hu[1], Yan Chen[1], Zhigang Wu[1], Sunjeet Kumar[1], Pengfei Duan[3], Lunguang Yao[3] and Hongwei Hou[1]**

[1] The State Key Laboratory of Freshwater Ecology and Biotechnology, The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Wuhan, China, [2] Centre for Plant Integrative Biology, University of Nottingham, Nottingham, United Kingdom, [3] Collaborative Innovation Center of Water Security for Water Source Region of Mid-line of South-to-North Diversion Project, College of Agricultural Engineering, Nanyang Normal University, Henan, China

Mercury (Hg) is a toxic heavy metal that can alter the ecological balance when it contaminates aquatic ecosystems. Previously, researchers have used various *Lemnaceae* species either to monitor and/or remove heavy metals from freshwater systems. As Hg contamination is a pressing issue for aquatic systems worldwide, we assessed its impact on the growth of three commonly species of *Lemnaceae* - *Lemna gibba* 6745, *Lemna minor* 6580 and *Spirodela polyrhiza* 5543. We exposed plants to different concentrations of mercuric chloride ($HgCl_2$) and monitored their growth, including relative growth rate, frond number (FN), and fresh weight (FW). These data were coupled with measurements of starch content, levels of photosynthetic pigment and the activities of antioxidant substances. The growth of all three lines showed significant negative correlations with Hg concentrations, and starch content, photosynthetic pigment, soluble protein and antioxidant enzymes levels were all clearly affected. Our results indicate that the *L. gibba* line used in this study was the most suitable of the three for biomonitoring of water contaminated with Hg. Accumulation of Hg was highest in the *S. polyrhiza* line with a bioconcentration factor over 1,000, making this line the most suitable of the three tested for use in an Hg bioremediation system.

Keywords: duckweed, mercuric chloride, toxicity test, growth indices, chemical composition, biomonitoring, bioremediation

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***Correspondence:**
Hongwei Hou
houhw@ihb.ac.cn

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INTRODUCTION

Mercury (Hg) is a toxic heavy metal element (Nieboer and Richardson, 1980; Fitzgerald and Clarkson, 1991). It can have devastating effects on organisms as well as on the whole environment when it contaminated aquatic ecosystems (Nagajyoti et al., 2010). Both natural and anthropogenic sources cause the accumulation of Hg in aquatic ecosystems. Natural sources include geologic parent material, rock outcroppings, wind-blown dusts, volcanic eruptions, marine aerosols and forest fires whilst anthropogenic sources include mining, coal burning and unsafe disposal of industrial solid/liquid wastes. Modern industrialization and urbanization led to the releases of Hg into ecosystems throughout the world (Sznopek and Goonan, 2000; Kolker et al., 2006; Larssen, 2010). A survey published in 2016 revealed that manufacturing activities in China released, 633 t of Hg emissions to the air, 84 t to water and 651 t to the land (Hui et al., 2017). This increasing contamination of Hg has led to a substantial accumulation within organisms. For example, a recent study reported high levels of methylmercury (MeHg) in the Bohai Sea, China, with some samples exceeding the Grade I limit established in China's seawater quality standard (50 ng/L) (Tong et al., 2017). Based on assessment of Hg contamination in China's coastal waters, MeHg concentrations in human blood were predicted to be between 1.37 and 2.77 mg/L for pregnant women and 0.43–1.00 mg/L for infants (Tong et al., 2017). Such levels affect human health and necessitate restrictions on seafood in the diet. This is a global problem and high Hg accumulation has even been observed in bats in the United States (Korstian et al., 2017). Discharge of industrial waste can also cause rapid increases in Hg. For example, discharge into a reservoir at the lower Ebro River in Catalonia (Spain) resulted in accumulation of Hg 20 times higher than the typical local concentration. Consequently, the total Hg (THg) and methylmercury (MeHg) content in zebra mussels collected in near sites were significantly elevated (Carrasco et al., 2008).

As Hg has such serious effects, considerable work has been done to minimize its discharge into both drinking and wastewater systems, and to maintain Hg levels below an established threshold (Ritter and Bibler, 1990). However, as Hg accumulates within the environment it is necessary to remove it from contaminated water bodies even though this is an expensive process (Pérez-Sanz et al., 2012). Whilst conventional methods such as ion exchange, membrane filtration, chelate precipitation, precipitation/adsorption are effective for aquatic ecosystems, the high costs often prevent their widespread deployment (Jeon and Park, 2005; Unlü and Ersoz, 2006; Wu et al., 2007). Therefore, there is a great need for alternative more cost—efficient methods to evaluate the contamination of Hg in aquatic ecosystems and to remediate these systems.

Plant based bioassays offer an attractive low cost solution to determine the effects and hazards of certain pollutants or environmental factors (Singh et al., 2007) and can provide convenient guidance for biomonitoring and bioremediation (Lewis and Wang, 1997; Roussel et al., 2000). Such processes rely on plants that have ability to accumulate certain substance (Tangahu et al., 2011). Recently there has been great interest in the use of duckweeds for both biomonitoring and bioremediation. Duckweeds are a small group of free-floating aquatic plants belonging to the Lemnaceae family. Members are commonly found in freshwater habitats such as ponds, lakes, ditches and rice paddies (Landolt, 1986). Due to a suite of properties including, their rapid growth rate, their ease of cultivation, the direct contact that they have with the water, their ability to adapt to environmental changes and their significant potential for both metal and nutrient uptake, duckweeds are becoming an attractive group of plants in various biotechnological applications (Lemon et al., 2001; Appenroth et al., 2013). Although the family contains five genera with 37 species, three species: *Lemna gibba*, *Lemna minor* and *Spirodela polyrhiza* have been studied extensively (Appenroth et al., 2013; Borisjuk et al., 2015; Forni and Tommasi, 2015). The use of duckweeds in such assays has become so widespread that standardized guidelines have been established to evaluate metal toxicity as well as removal of metal contaminants (Day and Saunders, 2004; Reinhold and Saunders, 2006; Tront et al., 2007). For example, ISO 20079 and OECD protocols provide detailed descriptions on the determination of toxicity effect of certain substances or polluted water on *L. gibba* or *L. minor* (Zayed et al., 1998; ISO 20079, 2005; OECD, 2006).

Numerous studies have already been conducted to assess the toxic effect of heavy metals on different duckweed species (Lakatos et al., 1993; Lahive et al., 2011; Leblebici and Aksoy, 2011; Appenroth et al., 2013; Gür et al., 2016). Such studies have proved instrumental in exploring the possibility of utilizing duckweeds as either biomarkers or in bioremediation. Whilst there is a substantial body of evidence assessing the effects of toxicity on duckweed growth of many elements, studies investigating the effect of mercury on duckweed growth are more limited. Li et al. (2011) have reported that water comprising inorganic and organic mercury at the concentration of 12.0 and 50.0 μg/L showed considerable reduction in the concentration of Hg after 40 min treatment with powdered *L. minor*. This processes resulted in treated water that was below both the maximum permitted concentration of Hg in drinking water (1.0 μg/L) and the permitted discharge limit of wastewater (10.0 μgL/L) set by China and USEPA. A comparative study showed that antioxidative enzymes can be activated within 24 h exposure to Hg although these enzymes are activated at lower Hg concentrations in *L. gibba* than *L. minor* (Varga et al., 2013). However, to date there has been no extensive study providing side-by-side comparisons of different duckweed species under various Hg concentrations.

Collectively these studies provide a detailed understanding about the impact of several potential contaminants on duckweed growth. This information could be exploited to produce new methods for biomonitoring or remediation, for example by those involved in environmental management, risk assessment and policy development. However, to fully exploit such a system for biomonitoring and bioremediation of Hg, several areas need to be addressed, these include (1) Establishing a quantitative description of toxic effect of Hg on duckweeds within a specific time range, (2) Observation of the specific response of duckweeds to Hg stress, (3) Comparison of Hg absorption and uptake by

different duckweeds, and (4) Identification of suitable duckweeds for Hg biomonitoring and bioremediation.

Within the biosphere, Hg is cycled between three oxidation states of Hg (0, I, and II; Barbosa et al., 2001). The majority of Hg exists in the form of inorganic mercuric salts ($HgCl_2$, $Hg(OH)_2$, HgS) and organomercurics (MeHg) (USEPA, 1997). Ionic mercury (Hg^{2+}) is the predominant form that can be absorbed and taken up by plants (Han et al., 2006) and therefore frequently accumulates in aquatic organisms (Pan and Wang, 2004). Mercuric chloride ($HgCl_2$) was used in this study since dissolves in water with relative ease. The toxicity of chlorine ions from $HgCl_2$ was not considered in this study because of its high content in cultivation medium and its negligible toxic effect compared to Hg. Lines of three duckweed species (*L. gibba*, *L. minor,* and *S. polyrhiza*) were chosen based on their widespread distribution and applicability for a toxicology experiment.

These three lines were grown under different concentrations of Hg, and growth assessed using existing methodology defined by the ISO 20079 guidelines. In addition we assayed other parameters to measure fitness including starch content, photosynthetic pigment, levels of antioxidant substances and Hg accumulation. Based on these data, we propose that different duckweed lines that can fulfill different roles in both biomonitoring and bioremediation of aquatic ecosystems contaminated with Hg.

MATERIALS AND METHODS

Duckweeds Culture and Toxicity Tests

Spirodela polyrhiza (L.) Schleid (5543) was collected from East Lake (N 30°32′, E 114°21′) at the city of Wuhan, Hubei Province, China. *Lemna minor* L. (6580 Harrington, Bergen Co., NJ, USA) and *Lemna gibba* L. (6745 Jacksonville, Tuolumn Co., CA, USA; Bog et al., 2010) were a gift from Prof. Hai Zhao, Chengdu Institute of Biology, Chinese Academy of Sciences. Current toxicity tests were conducted according to the ISO 20079 criteria (ISO 20079, 2005) using modified *Steinberg* medium (Naumann et al., 2007). The composition of *Steinberg* medium was 3.46 mM KNO_3, 1.25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.66 mM KH_2PO_4, 0.072 mM K_2HPO_4, 0.41 mM $MgSO_4{\cdot}7H_2O$, 1.94 μM H_3BO_3, 0.63 μM $ZnSO_4{\cdot}7H_2O$, 0.18 μM $Na_2MoO_4{\cdot}2H_2O$, 0.91 μM $MnCl_2{\cdot}4H_2O$, 2.81 $FeCl_3{\cdot}6H_2O$, 4.03 mM EDTANa2. Pre-cleaned 500 mL Erlenmeyer flasks containing 100 mL sterilized *Steinberg* medium (pH 5.5 ± 0.2) were supplemented with 7 different concentrations of $HgCl_2$ (Sigma-Aldrich, purity >99%)—0, 0.25, 0.5, 1, 2, 4, 8 mg/L or 0, 0.92, 1.84, 3.68, 7.37, 14.73, 29.47 μM. This concentration gradient was based on preliminary data from a 24 h acute toxicity test and set based on a geometric scale. 12 axenic fronds (3 colonies each for *L. gibba* and *L. minor*, 4 colonies for *S. polyrhiza*) per flask were added to the culture solution. Colonies were selected with roughly equal sizes from the pre-cultivated axenic stocks and used to inoculate cultures. All toxicity experiments were conducted at 24 ± 2°C under continuous white light at 85 μmol $m^{-2}s^{-1}$ and lasted for 7 days. Experiments were performed in triplicate to allow statistical analyses of results.

Growth Measurements

The frond number (FN) (all visible fronds) in each flask was recorded daily throughout the experiment. The fresh weight (FW) was recorded at the beginning and end of the experiment. The calculations of growth rate followed standardized procedures described in ISO 20079 criteria (ISO 20079, 2005).

Chemical Composition of Duckweeds

To determine soluble protein and antioxidative enzymes, fresh plant materials (0.05 g) were homogenized in ice with 0.5 mL phosphate saline buffer (pH 7.4, 0.1 M) using a glass homogenizer. Homogenized samples were centrifuged at 3,500 rpm for 20 min. This supernatant was used to determine the content of soluble protein and activities of antioxidative enzymes (total superoxide dismutase, catalase, peroxidase) using commercially available test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Li et al., 2013; Yan et al., 2013).The absorbance of the supernatant was detected by BioDrop uLite (80-3006-51) under visible light at different wavelengths.

Photosynthetic pigments of duckweeds were extracted in 80% chilled acetone in the dark and estimated as described by Porra et al. (1989). Starch extraction and quantification were done according to the method described by Magel (1991). Starch was extracted with 18% (w/v) HCl. Detection was conducted using 0.5% (w/v) KI and 0.25% (w/v) I_2 and measured at 605 nm and 530 nm. To determine the Hg content in duckweeds, plant material was dried at 75°C and digested with 10 ml concentrated HNO_3 acid with the help of microwave digestion system (Anton paar, Multiwave 3000). Digested samples were diluted up to 10 ml with ultra-deionized water. Final concentrations of $K_2Cr_2O_7$ and HNO_3 of the samples were adjusted to be within 0.05% (M/V) and 0.05% (V/V) respectively. The residual level of Hg in each sample was measured using Atomic Fluorescence Spectrometer (Analytikjena, ContrAA 700) at the Center of Analysis and Test Center of Wuhan University.

Statistical Analysis

All measurements were conducted using independent plant samples. The SPSS statistical programme (version 18.0) was used for statistical analysis (including variance tests, determining EC50 values and the corresponding 95% confidence intervals, probit regression analysis, specifying the corresponding fitting coefficient (R^2), and one-way ANOVA analysis). Tukey tests were performed to determine the significance differences among treatments. Values presented in this manuscript are means ± SDs.

RESULTS

Growth of Duckweeds

To provide a baseline for subsequent toxicity experiments the growth of three duckweed lines was first measured in cultures without Hg treatment. To ensure the validity of this study the number of fronds in control groups should have a 7-fold increase by the end of 7 days experiment as specified in the ISO 20079 criterion (ISO 20079, 2005). After exponential

growth for 7 days, the FN of *L. gibba*, *L. minor*, *S. polyrhiza* increased more than 7 times ($R^2 = 0.9966$, 0.993, and 0.9857, respectively) (**Figures 1A–C**) indicating the validity of this study. No significant difference was observed among these three duckweed lines from days 0 to 7 ($P > 0.05$). The relative growth rate (RGR) based on FN was defined as the average specific growth during a certain period, and this was used to assess the growth of three duckweed lines. The RGR of *L. gibba*, *L. minor* and *S. polyrhiza* in control conditions after 7 days were 0.28 ± 0.003, 0.29 ± 0.007, and 0.28 ± 0.006 per day, respectively (**Figures 1D–F**). No significant difference was observed among three duckweed lines at days 1, 3, 5, 7 ($P > 0.05$).

Effect of Hg on the Growth of Duckweeds

The FN and FW of duckweeds grown in media supplemented with 0.25, 0.5, 1, 2, 4, and 8 mg/L Hg were used to evaluate the toxic effect of Hg on the growth of duckweeds. The FN of all three duckweed lines showed a close relationship with Hg concentration, and in all Hg treatments FN increased with time (**Figures 1A–C**). The three treated lines showed significant differences in FN when compared with untreated plants at all Hg concentrations analyzed (0.25, 0.5, 1, 2, 4, and 8 mg/L) ($P < 0.05$). No significant difference in FN was observed between *L. gibba* and *L. minor* from days 3 to 7 ($P > 0.05$). However at day 7, the FN of *S. polyrhiza* showed significant differences with *L. gibba* and *L. minor* at 8 mg/L Hg ($P < 0.05$), indicating that *S. polyrhiza* was more resistant to Hg. No significant difference was observed between the 4 mg/L and 8 mg/L treatments ($P > 0.05$) on FN for the three lines, suggesting that all three lines were equally affected at 4 and 8 mg/L levels of Hg.

The RGR values of the three duckweed lines also changed with increasing Hg level and exposure time (**Figures 1D–F**). In the *L. gibba* line, there was no significant difference between RGR values at 8 mg/L and 4 mg/L treatment from days 1 to 7 ($P > 0.05$) but significant differences were observed between 8 mg/L and 0, 0.25, 0.5, 1 and 2 mg/L treatments at days 3, 5, and 7 ($P < 0.05$;

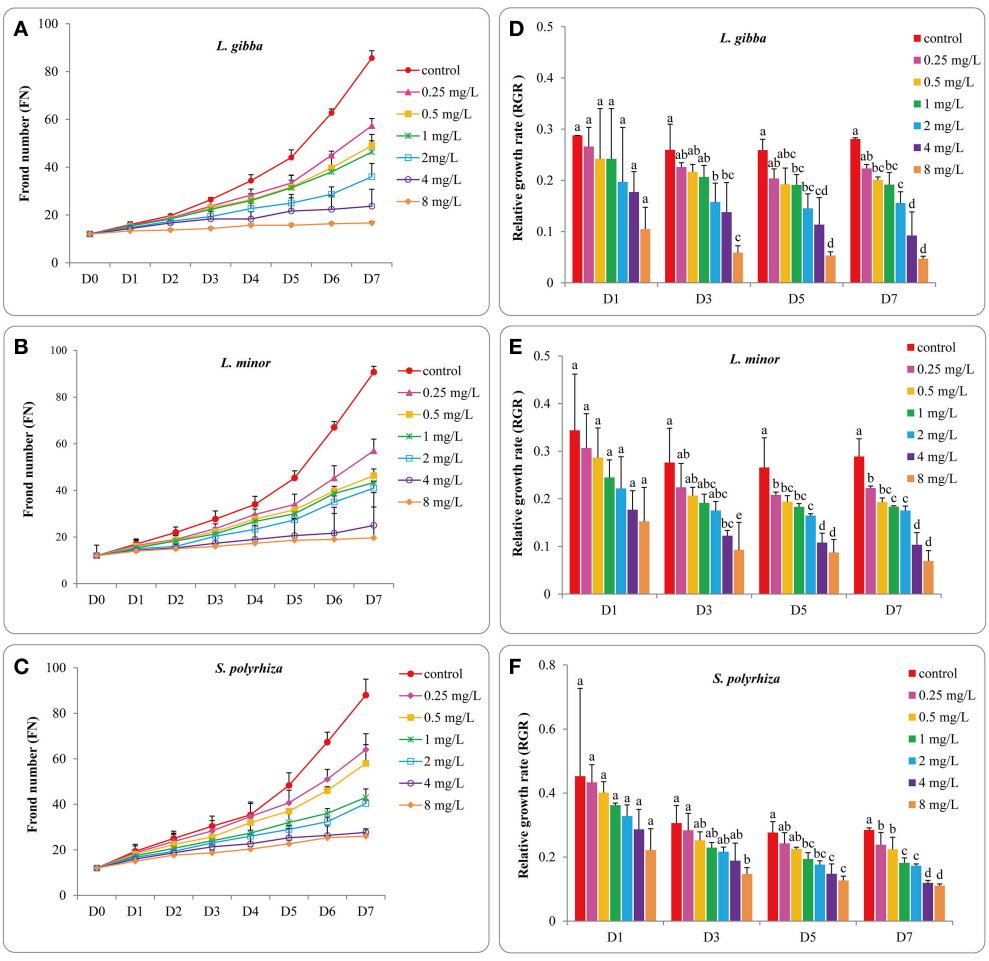

FIGURE 1 | Effects of different concentrations of Hg on the frond number and the corresponding relative growth rates of *L. gibba* **(A,D)**, *L. minor* **(B,E)** and *S. polyrhiza* **(C,F)** at 1, 3, 5, 7 days. The letters (a, b, c, d, e) on the column graphs indicated Tukey tests analyses results among different Hg treatments at 1, 3, 5, 7 days in the same duckweed. The same letters indicated no significant differences and different letters indicated significant difference among treatments. Error bars indicated standard deviation.

Figure 1D). In *L. minor*, no significant difference was observed between the RGR values at 8 mg/L and 0, 0.25, 0.5, 1, 2, and 4 mg/L treatments at day 1 ($P < 0.05$; **Figure 1E**). The RGR value at 8 mg/L began to show significant differences with 0, 0.25, 0.5, 1, and 2 mg/L Hg treatments ($P < 0.05$). In *S. polyrhiza*, the RGR value at 8 mg/L treatment was not significantly different from 1, 2, and 4 mg/L till day 5 ($P > 0.05$; **Figure 1F**) indicating that the negative influence on RGR rate of *S. polyrhiza* was lower than for *L. minor* and *L. gibba*.

In order to allow for the accurate quantification of the inhibitory effect of Hg, the percent inhibition of growth rates (Ir) of three lines was estimated based on FN. This was used to determine the EC50 (half maximal effective concentration) values as well as a dose-response relationship as has been described in the ISO 20079 guidelines (ISO 20079, 2005). When the three duckweed lines were grown at 4 mg/L Hg, the growth inhibition reached 50% at day 3 in *L. gibba*, day 5 in *L. minor* and day 7 in *S. polyrhiza*. The growth inhibition exceeded 50% within 24 h of exposure to 8 mg/L Hg for all three duckweed lines. The highest EC50 values of *L. gibba*, *L. minor* and *S. polyrhiza* at day 1 were 4.4, 5.0, and 7.5 mg/L, respectively (**Table 1**). Among the three duckweed lines, the highest EC50 value was obtained in *S. polyrhiza* and the lowest EC50 value was obtained in *L. gibba*. These results indicate that the *S. polyrhiza* line had the highest tolerance to Hg when compared with the *L. minor* and *L. gibba* lines.

The FW of treated duckweeds was measured at the end of experiment (day 7) to evaluate the RGR values of different Hg treatments (**Figure 2**). The RGR based FW of all three lines declined sharply with Hg treatment and even negative growth rates were observed at 4 and 8 mg/L treatments. All of the RGR values based upon FW at 4 and 8 mg/L treatments were found to be significantly different from those at 0, 0.25, 0.5, 1, and 2 mg/L treatments. Significant differences between 4 and 8 mg/L treatment were also observed in *L. minor* ($P < 0.05$) while no significant difference was observed in *L. gibba* and *S. polyrhiza* ($P < 0.05$).

TABLE 1 | Toxicity assessment of dose-response regression equations for *L. gibba*, *L. minor* and *S. polyrhiza* under different Hg treatments.

Species	Exposure days	Regression equation	EC50 (mg/L)
L. gibba	1	y = 1.258lgx-0.806, $R^2 = 0.914$	4.4 (2.6, 11.7)
	3	y = 1.226lgx-0.625, $R^2 = 0.92$	3.2 (2.0, 6.8)
	5	y = 1.044lgx-0.38, $R^2 = 0.897$	2.3 (1.3, 5.0)
	7	y = 1.166lgx-0.291, $R^2 = 0.936$	1.8 (1.1, 2.9)
L. minor	1	y = 0.926lgx-0.641, $R^2 = 0.989$	4.9 (3.6, 7.7)
	3	y = 0.877lgx-0.442, $R^2 = 0.96$	3.2 (2.4, 4.7)
	5	y = 0.854lgx-0.384, $R^2 = 0.94$	2.8 (2.1, 4.1)
	7	y = 0.919lgx-0.266, $R^2 = 0.912$	1.9 (1.1, 3.8)
S. polyrhiza	1	y = 1.057lgx-0.928, $R^2 = 0.984$	7.5 (5.4, 12.3)
	3	y = 0.88lgx-0.766, $R^2 = 0.966$	7.4 (5.0, 13.4)
	5	y = 0.837lgx-0.608, $R^2 = 0.989$	5.3 (3.7, 9.0)
	7	y = 0.895lgx-0.454, $R^2 = 0.968$	3.2 (2.4, 4.7)

Effect of Hg on Antioxidant Protective Mechanism

The soluble protein content of the three lines increased with lower levels of Hg (to around 1 mg/L), but was decreased with higher concentrations of Hg (**Figure 3A**). The highest soluble protein contents reported were 4.40 ± 0.13 mg/g in *L. gibba*, 3.97 ± 0.25 mg/g in *L. minor* at 0.5 mg/L treatment and 5.92 ± 0.13 mg/g in *S. polyrhiz*a at 1 mg/L treatment. Significant differences were observed between the control and 0.25, 0.5, 1, and 2 mg/L treatments in *L. gibba* ($P < 0.05$). No significant difference was observed between the control and either 0.25 or 1 mg/L treatments in *L. minor* ($P > 0.05$) but 0, 0.25, and 1 mg/L treatments were significantly different from 0.5, 2, 4, and 8 mg/L treatments ($P < 0.05$). In the *S. polyrhiza* line, no significant difference was observed between the control and 0.25 mg/L treatment ($P > 0.05$), but the 0 and 0.25 mg/L treatments had significantly different values for soluble protein content from the 0.5, 1, 2, 4, and 8 mg/L treatments ($P < 0.05$).

The total superoxide dismutase (T-SOD) activities increased with Hg treatment in all three lines and reached a peak at 1 mg/L in *L. gibba*, 2 mg/L in *L. minor* and *S. polyrhiza*. The *L. gibba* line had 290.50 ± 5.59 U/mg protein, the *L. minor* line 380.59 ± 12.21 U/mg protein and the *S. polyrhiza* line 277.40 ± 19.16 U/mg protein (**Figure 3B**). No significant difference was observed between the control and 0.25, 0.5 mg/L treatments in the three

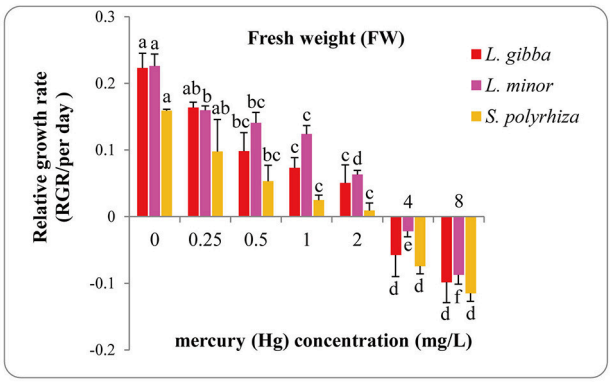

FIGURE 2 | Effects of different concentrations of Hg on the relative growth rates based on fresh weight (FW) of three duckweed lines. The letters (a, b, c, d) on the column graphs indicated Tukey tests analyses results among different Hg treatments in the same duckweed. The same letters indicated no significant differences and different letters indicated significant difference among treatments. Error bars indicated standard deviation.

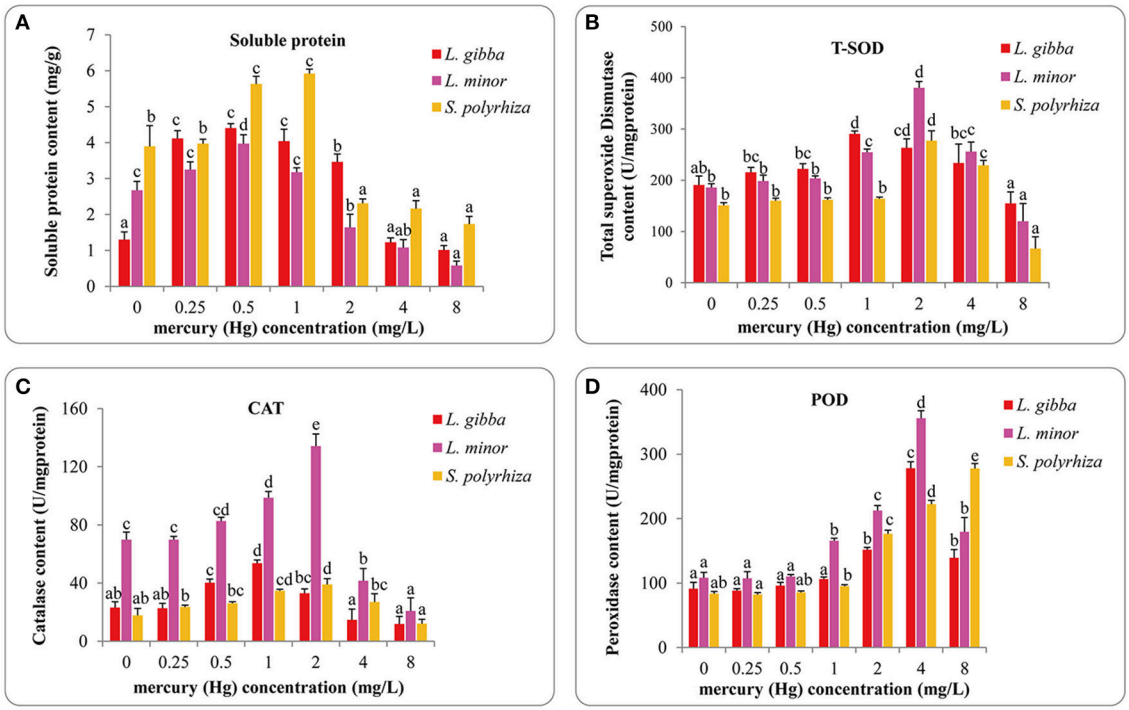

FIGURE 3 | Effects of different concentrations of Hg on the content of soluble protein **(A)** and activities of T-SOD **(B)**, CAT **(C),** and POD **(D)** of three duckweed lines. Indices were measured after 7 days treatment and calculated based on the fresh weight (FW). The letters (a, b, c, d, e) on the column graphs indicated Tukey tests analyses results among different Hg treatments in the same duckweed. The same letters indicated no significant differences and different letters indicated significant difference among treatments. Error bars indicated standard deviation.

lines ($P > 0.05$). However, the 0.25, 0.5 mg/L treatments were significantly different from 1 and 2 mg/L treatments ($P < 0.05$) in *L. gibba*. In *L. minor,* the 0, 0.25, and 0.5 mg/L treatments were significantly different from 1, 2, 4, and 8 mg/L treatments ($P < 0.05$). And in *S. polyrhiza*, only the 0, 0.25, and 0.5 mg/L treatments were significantly different from 2, 4 ,and 8 mg/L treatments ($P < 0.05$).

As well as investigating T-SOD activity, we also investigated variation in the levels of catalase (CAT) activity (**Figure 3C**). The highest levels observed were 53.64 ± 2.23 U/mg protein in *L. gibba* at 1 mg/L Hg treatment, 134.11 ± 8.42 U/mg protein in *L. minor* and 39.11 ± 3.91 U/mg protein in *S. polyrhiza* at 2 mg/L Hg treatment. The lowest activities recorded at 8 mg/L treatment were 11.92 ± 5.16 U/mg protein in *L. gibba*, 20.86 ± 9.03 U/mg protein in *L. minor* and 12.17 ± 3.01 U/mg protein in *S. polyrhiza*. In *L. gibba,* there was no significant difference between the control and 0.25, 2, 4, and 8 mg/L treatments ($P > 0.05$). In *L. minor*, significant differences were observed between the control and 1, 2, 4, and 8 mg/L treatments ($P < 0.05$). And in *S. polyrhiza,* significant differences were only observed between control and 1, 2 mg/L treatments ($P < 0.05$).

We finally investigated variation in the levels of peroxidase activity (POD) (**Figure 3D**). We saw maximum POD level at 4 mg/L treatment in *L. gibba* and *L. minor,* and like the previous protective mechanisms the activity was reduced at higher concentrations. In contrast POD activity increased consistently in the *S. polyrhiza* line until the highest concentration tested (8 mg/L). This indicates that *S. polyrhiza* exhibits a constant response to high levels of Hg. The highest value obtained in *L. gibba, L. minor* and *S. polyrhiza* were 278.36 ± 9.93, 355.77 ± 11.85, and 277.94 ± 7.41 U/mg protein, respectively. No significant difference was observed within the control, 0.25 and 0.5 mg/L treatments of the three lines ($P > 0.05$). However, the 0, 0.25, and 0.5 mg/L treatments were significantly different from 2, 4, and 8 mg/L treatments in all three lines ($P < 0.05$). All of the POD activity measurements in 1 mg/L were significantly different from 2 and 4 mg/L treatments. However, there was no significant difference between 0.5 and 1 mg/L treatments in *L. gibba* and *S. polyrhiza* ($P > 0.05$). In *L. minor*, significant differences were observed between 1 mg/L and 0, 0.25, and 0.5 mg/L treatments ($P < 0.05$).

Chlorophyll Content

The influence of Hg on photosynthetic pigment content (chlorophyll *a*, chlorophyll *b* and chlorophyll *a*/*b*) of *L. gibba*, *L. minor,* and *S. polyrhiza* lines is shown in **Figures 4A–C**. The pigment content in *L. gibba* was negatively correlated with Hg exposures and the highest values of chlorophyll *a*, chlorophyll *b* and chlorophyll *a*/*b* were 9.09 ± 0.18 mg/g (FW), 4.43 ± 0.22 mg/g (FW), and 2.78 ± 0.11 mg/g (FW) in control groups. In *L. minor*, the Chlorophyll *a* content reached to its maximum at 0.25 mg/L Hg treatment of 8.53 ± 0.37 mg/g (FW), while the

maximum content of chlorophyll *b* was 3.80 ± 0.49 mg/g (FW) at 0.5 mg/L treatment. In *S. polyrhiza*, the maximum chlorophyll *a* content was 8.89 ± 0.65 mg/g (FW) at 1 mg/L treatment, whilst chlorophyll *b* content reached to the maximum value of 5.79 ± 1.18 mg/g (FW) in the 0.5 mg/L treatment. The highest ratios of chlorophyll *a*/*b* were 2.78 ± 0.11, 3.17 ± 0.14 for *L. gibba* and *L. minor* at 1 mg/L Hg treatment and 3.65 ± 0.69 for *S. polyrhiza* in the 2 mg/L treatment.

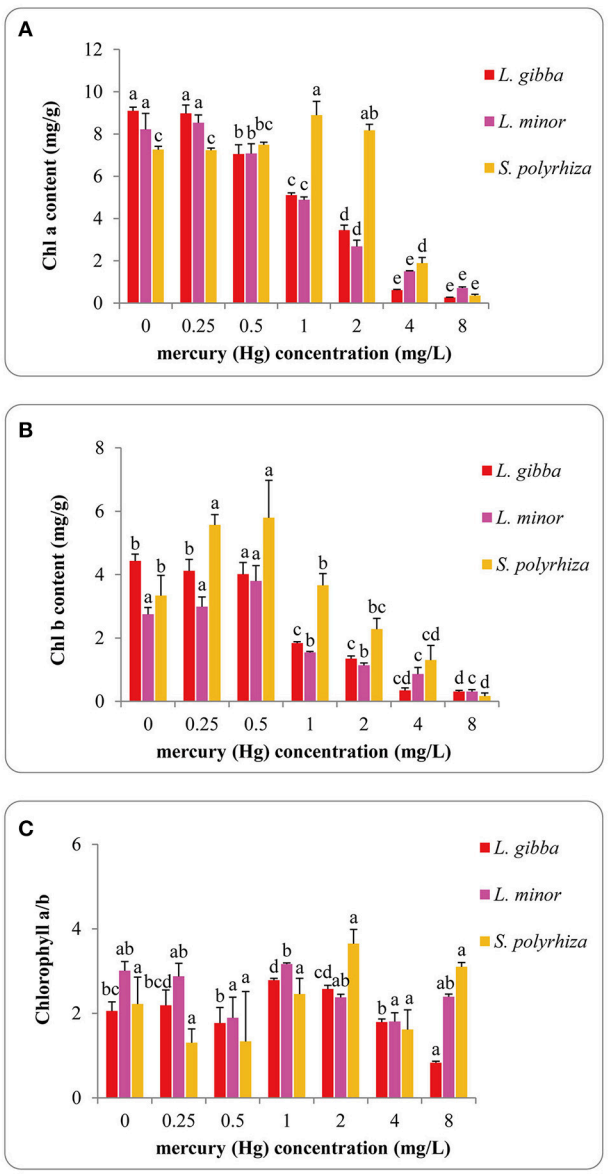

FIGURE 4 | Effects of different concentrations of Hg on the content of Chlorophyll a **(A)**, Chlorophyll b **(B)** and Chlorophyll a/b **(C)** of three duckweed lines. The pigment content was measured after 7 days Hg treatment and calculated based on the fresh weight (FW). The letters (a, b, c, d, e) on the column graphs indicated Tukey tests analyses results among different Hg treatments in the same duckweed. The same letters indicated no significant differences and different letters indicated significant difference among treatments. Error bars indicated standard deviation.

The chlorophyll *a*, chlorophyll *b* and chlorophyll *a*/*b* content of *L. gibba* were significantly different between the control and 1, 2, 4, and 8 mg/L treatments ($P < 0.05$). No significant difference was observed between the control and 0.25 mg/L Hg treatment ($P > 0.05$). In *L. minor*, chlorophyll *a* and chlorophyll *b* content in control samples were significantly different from 0.5, 1, 2, 4, and 8 mg/L treatments ($P < 0.05$). In *S. polyrhiza*, chlorophyll *a* content in the control was significantly different from 1, 2, 4, and 8 mg/L treatments ($P < 0.05$). There were significant differences between the chlorophyll *b* content of the control and 0.5, 4, 8 mg/L treatments ($P < 0.05$). No significant difference was observed among all the treatments of chlorophyll *a*/*b* ($P > 0.05$).

Effects of Hg on Starch Content

Starch content increased with increasing Hg concentration in all three lines and reached the highest in the 2 mg/L treatment with the maximal values of 27.42 ± 0.24, 38.16 ± 0.86, and 45.24 ± 3.86 (% DW) for *L. gibba*, *L. minor,* and *S. polyrhiza*, respectively (**Figure 5**). In every treatment the starch content of *S. polyrhiza* was higher than that of *L. gibba* and *L. minor*. Significant differences were observed between control and 0.25, 0.5, 1, 2, and 8 mg/L treatments in *L. gibba* ($P < 0.05$). In *L. minor*, significant differences were observed among all Hg treatments ($P < 0.05$). No significant difference was observed between the control and 0.25 mg/L treatment in *S. polyrhiza* ($P > 0.05$), whilst the control was significantly different from 0.5, 1, 2, 4 mg/L treatments ($P < 0.05$).

Mercury Accumulation in Duckweed

Mercury accumulation was monitored in all three lines at the various concentrations (**Figures 6A,B**). In all lines, as expected Hg accumulation increased sharply with Hg treatments and reached the maximum in the 8 mg/L treatment (**Figure 6A**). The highest values in *L. gibba, L. minor,* and *S. polyrhiza* were 1.74 ± 0.02 mg/g (DW), 2.55 ± 0.004 mg/g (DW), and

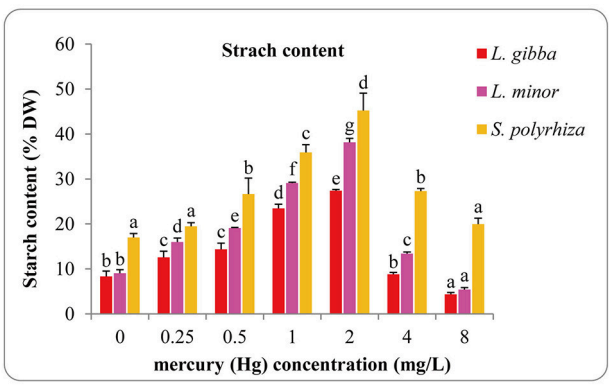

FIGURE 5 | Effects of different concentrations of Hg on starch content of three duckweed lines. The starch content was measured after 7 days and calculated based on the dry weight (DW). The letters (a, b, c, d, e, f) on the column graphs indicated Tukey tests analyses results among different Hg treatments in the same duckweed. The same letters indicated no significant differences and different letters indicated significant difference among treatments. Error bars indicated standard deviation.

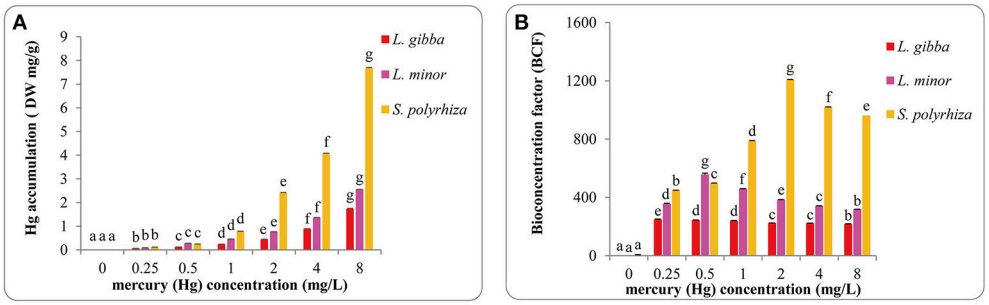

FIGURE 6 | Hg accumulation **(A)** and bioconcentration factors (BCF) of Hg accumulation **(B)** of three duckweed lines under different concentrations of Hg treatments. Hg accumulation was measured after 7 days and calculated based on the dry weight (DW).The letters (a, b, c, d, e, f, g) on the column graphs indicated Tukey tests analyses results among different Hg treatments in the same duckweed. The same letters indicated no significant differences and different letters indicated significant difference among treatments. Error bars indicated standard deviation.

7.70 ± 0.01 mg/g (DW) respectively, showing that the *S. polyrhiza* line can accumulate substantially more Hg. Hg accumulation was significantly different among every treatments in the three lines ($P < 0.05$). *S. polyrhiza* clearly accumulated the highest Hg quantity followed by *L. minor* and *L. gibba*.

It is important to relate the concentration of certain elements within an organism to the concentration in the environment where the organism exists, and this can be done by measuring the bioconcentration factor (BCF). We calculated BCF values of Hg accumulation in all treatments (**Figure 6B**). In *L. gibba*, BCF dropped from the maximum of 249.42 ± 2 in the control to 217.47 ± 2 in the 8 mg/L treatment. In *L. minor*, the value increased to the highest at 557.52 ± 8 in the 0.5 mg/L treatment and then decreased in treatments between 1 and 8 mg/L. In *S. polyrhiza*, the highest value obtained was 1208.67 ± 2.41 in the 2 mg/L treatment. No significant difference was observed between treatments of 0.5 and 1 mg/L as well as 2 and 4 mg/L in *L. gibba* ($P > 0.05$). However in *L. gibba*, the BCF values at 0.5, 1, 2, and 4 mg/L treatments showed significant differences from 0.25 and 8 mg/L treatments ($P < 0.05$). BCF values in *L. minor* and *S. polyrhiza* were significantly different among every treatment ($P < 0.05$).

DISCUSSION

L. gibba, *L. minor,* and *S. polyrhiza* Are Ideal Plants for Hg Biomonitoring and Bioremediation

Previous studies have reported that duckweeds are highly sensitive to a broad range of pollutants and show multivariable stress responses when compared with other aquatic macrophytes (Cedergreen et al., 2004). Their characteristics of simple structure, minute size, rapid multiplication and easy cultivation make them ideally suited for use as bioindicators in aquatic habitats (Wang, 1990; Forni, 2014). In such assays, the use of visible parameters, such as total frond number make it possible to assess contamination in a direct and rapid manner. Easily measurable stress response parameters such as starch content, or photosynthetic pigment can also be used to provide effective toxicological evaluations (USEPA, 1996; Marwood et al., 2001; Baumann et al., 2008; Pietrini et al., 2015) and individual contaminants may elicit a more specific response in such assays. In this study, we investigated three lines corresponding to different duckweed species using a combination of growth and chemical assays. We report serious growth effects at high Hg levels (4 and 8 mg/L), and consistent with other current research, we suggest that duckweeds provide a suitable system for biomonitoring of Hg in waters contaminated with less than 4 mg/L Hg level.

Our results indicated that out of the three lines analyzed, *L. gibba* was more suitable for Hg biomonitoring than *L. minor* and *S. polyrhiza* as it displayed the highest sensitivity to Hg. It should be noted that these results are specific for Hg, and different sensitivities of duckweeds have been reported for other heavy metals. For example, Lahive et al. (2011) reported that *Landoltia punctata*, *L. minor*, *Wolffia brasiliensis* and *L. gibba* had distinct sensitivity to zinc sulfate with *L. punctata* being the most sensitive. Gür et al. (2016) reported that *L. minor* was more sensitive to boron (B) than *L. gibba* while *L. gibba* showed a wider range of responses for B than *L. minor* in biomonitoring. Lakatos et al. (1993) also reported that *L. minor* was more sensitive than *L. gibba* to both copper and Bonion biocide exposure. According to Leblebici and Aksoy (2011) the ability of *L. minor* to extract lead from the surrounding environment was more effective than *S. polyrhiza* while *S. polyrhiza* was more sensitive than *L. minor*. According to Appenroth et al. (2010) *S. polyrhiza* was more sensitive to nickel than *L. minor*. These findings demonstrate distinctly that the outcome of biomonitoring assessments of pollutants using duckweeds is both highly species dependent, but also dependent on the contaminant. Therefore, selection of duckweed species for toxicity assessment should be done carefully.

Our results indicated BCF was a more effective measurement than Hg accumulation to provide accurate quantification of heavy metal uptake in duckweeds. It has been previously been shown for a variety of plants that higher BCF values indicate a stronger ability for metal uptake (Salt et al., 1995; Zayed et al., 1998). Accordingly, in our experiments, the *S. polyrhiza* line had a relatively high BCF value. According

to Zayed et al. (1998), a plant with a BCF of over 1,000 can be considered as good accumulator for the compound of interest. Therefore, the results obtained here suggest that the *S. polyrhiza* line used in this study could potentially be used for Hg remediation in aquatic ecosystems. Whilst both *L. gibba* and *L. minor* are unsuitable for Hg bioremediation, these lines used in this study would be more effective for use in Hg biomonitoring.

Antioxidant Substances, Photosynthetic Pigment and Starch Content Are Ideal Biomarkers in Toxicity Assessments

A range of protective substances, including antioxidant enzymes (SOD, CAT, POD), soluble proteins, flavonoids, and other phenolics have been shown to accumulate in plants as an adaptive mechanism for coping with certain stresses (Horling et al., 2003; Mittler et al., 2004; Sharma and Dietz, 2009; Varga et al., 2013). Each enzyme has a different role to play in plant protection; SOD converts superoxide to H_2O_2 (Mishra et al., 2006), whilst CAT and POD breakdown the H_2O_2 (Scandalios et al., 1994). Some soluble proteins also form an important antioxidant constituent that is needed to maintain metabolism (Singh and Tewari, 2003). Flavonoids and other phenolics may constitute a secondary antioxidant system when antioxidant enzymes are depleted, and specifically counter the oxidant load in the vacuole (Agati et al., 2012).

In this manuscript we report that the three duckweeds tested resist metal-stress at low Hg treatments (0.25, 0.5,1 mg/L) by accumulating soluble protein within their tolerance range. In all cases the soluble protein content decreased at high Hg levels (2, 4, 8 mg/L). These findings are similar to the changes in soluble protein content in *L. minor* reported under treatments with between 0 and 500 μM $CdCl_2$ (Razinger et al., 2008). However, Varga et al. (2013) have reported direct reduction of protein content in *L. minor* and *L. gibba* after 24 h exposure to different Hg and Cd concentrations (0, 100, 200, 300, 400, 500, and 600 μM), most likely due to excessive stress in the short-term. The inability to synthesize protein at high Hg concentrations (2, 4, 8 mg/L) might be due to several reasons, such as shortage of energy, carbohydrates or a reduced level of nutrients essential for protein synthesis, such as Mg and K (Mazhoudi et al., 1997; Gardea-Torresdey et al., 2004; Wang et al., 2008), increased protease activity (Palma et al., 2002) or structural changes induced by DNA damages or damage to the photosynthetic system (Ates et al., 2004; Gardea-Torresdey et al., 2004). The changes that we observed in the levels of SOD, CAT and POD in Hg-treated duckweeds were similar to findings in other studies, following the oxidative stress by Hg treatment in wheat (Sahu et al., 2012) and into ammonium-induced oxidative stress in *L. minor* (Huang et al., 2013). Our studies revealed that the *S. polyrhiza* line had a higher tolerance to Hg than both the *L. gibba* and *L. minor* lines, since antioxidant enzyme activities in this line decreased at relatively high Hg levels and POD content increased until 8 mg/L.

It has been reported that heavy metal stress affects pigment content in many plant species (Prasad et al., 2001; Hou et al., 2007; Perreault et al., 2013; Sree et al., 2015). In this study, the pigment content of *L. minor* and *S. polyrhiza* showed an initial increase, followed by a significant decrease under different Hg treatments, and this is likely due to the combinational effect of photoprotection and antioxidative production as reported by Lalau et al. (2015). *L. gibba* was more sensitive to Hg than both *L. minor* and *S. polyrhiza,* and accordingly we observed that the pigment content decreased under Hg stress from 0.25 to 8 mg/L. Moreover, the chlorophyll *a* content decreased sharply at high Hg concentrations (from 2 to 8 mg/L) indicating that chlorophyll *a* was a more sensitive biomarker than chlorophyll *b* and chlorophyll *a*/*b*, a phenomenon that has been observed in other studies (Hou et al., 2007; Appenroth et al., 2010). Starch accumulation has also been identified as a protective mechanism in duckweeds to overcome adverse environmental conditions (Sree and Appenroth, 2014). Accordingly, we observed an increase in the starch content of all three duckweed lines when exposed to low Hg treatments (0.25, 0.5, 1, 2 mg/L). In a similar manner to other research, we observed that the inhibition of growth also reduced the demand for carbohydrates and consequently reduced the starch content (Appenroth et al., 2010). Moreover, we observed substantial reduction in pigment content when duckweeds were exposed to high Hg treatments (4, 8 mg/L) and this is likely to lead to insufficient supplies of carbohydrates. As a result the starch reserves are likely to have been used to fulfill the carbohydrate demand (Sree et al., 2015).

Collectively our data show that different concentrations of mercuric chloride ($HgCl_2$) affected the growth, photosynthetic pigment, starch content and antioxidant system of *L. gibba*, *L. minor* and *S. polyrhiza.* When Hg concentrations are increased, we observed reduction of RGR, FN, FW in all three lines. As Hg concentrations are altered, the levels of starch, photosynthetic pigment and soluble protein are all modulated. Moreover, at these concentrations, increasing the synthesis of SOD, CAT, and POD appears to enhance the antioxidant protective mechanisms. However, at very high levels of Hg stress, these mechanisms have been inhibited. Our analysis of EC50 values indicated that the *L. gibba* line was more sensitive to Hg toxicity than both *L. minor* and *S. polyrhiza*. Therefore it can be concluded that *L. gibba* is more efficient in Hg biomonitoring than *L. minor* and *S. polyrhiza*. *S. polyrhiza* shows a very high BCF (over 1,000) and based on this we propose that the *S. polyrhiza* line used in this study has a great potential for bioremediation of Hg contaminated aquatic ecosystems.

ETHICS STATEMENT

This manuscript did not include human subjects or animals. Therefore, ethics approval was not required.

AUTHOR CONTRIBUTIONS

HH designed and funded this project. JY and GL conducted the experiments. PH and SH cultivated plants and processed

sample treatments. YC and ZW performed statistical analysis and data processing. AB, SK, PD, and LY wrote the manuscript.

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Bacterial Production of Indole Related Compounds Reveals Their Role in Association Between Duckweeds and Endophytes

Sarah Gilbert[1], *Jenny Xu*[1], *Kenneth Acosta*[1], *Alexander Poulev*[1], *Sarah Lebeis*[2] *and Eric Lam*[1]*

[1] Department of Plant Biology, Rutgers University, New Brunswick, NJ, United States, [2] Department of Microbiology, University of Tennessee, Knoxville, TN, United States

Duckweed farming can be a sustainable practice for biofuel production, animal feed supplement, and wastewater treatment, although large scale production remains a challenge. Plant growth promoting bacteria (PGPB) have been shown to improve plant health by producing phytohormones such as auxin. While some of the mechanisms for plant growth promotion have been characterized in soil epiphytes, more work is necessary to understand how plants may select for bacterial endophytes that have the ability to provide an exogenous source of phytohormones such as auxin. We have isolated and characterized forty-seven potentially endophytic bacteria from surface-sterilized duckweed tissues and screened these bacterial strains for production of indole related compounds using the Salkowski colorimetric assay. Indole-3-acetic acid (IAA), indole-3-lactic acid (ILA), and indole produced by various bacterial isolates were verified by mass spectrometry. Using the Salkowski reagent, we found that 79% of the isolated bacterial strains from our collection may be capable of producing indole related compounds to various extents during *in vitro* growth. Of these bacteria that are producing indole related compounds, 19% are additionally producing indole. There is an apparent correlation between the type of indole related compound produced by a particular bacteria and the duckweed genus from which the bacterial strain is derived. These results suggest the possible association between different duckweed genera and endophytes that are producing distinct types of secondary metabolites. Understanding the role of indole related compounds during interaction between endophytes and the plant host may be useful to help design synthetic bacterial communities that could target specific or multiple species of duckweed in the future to sustainably enhance plant growth.

Keywords: auxin, *Wolffia*, duckweeds, indoles, Salkowski assay, endophytes

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***Correspondence:**
Eric Lam
eric.lam@rutgers.edu

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INTRODUCTION

Duckweeds are aquatic plant species that preferentially reproduce via asexual propagation and are found all over the world. Their fast growth rate and ability to remove excess nitrogen and phosphate pollutants makes them attractive for use in wastewater treatment (Korner and Vermaat, 1998). In addition, duckweed biomass can be harvested for biofuel production or as animal feed additives,

since strains with high levels of starch or protein can be identified (Cheng and Stomp, 2009). With the increasingly urgent need for low cost wastewater treatment, alternative energy sources, and increased food supply to support the growing world population, duckweed is a promising area of research that can have an impact on these challenges. However, large-scale production of duckweed continues to be a limiting factor for growing duckweed at wastewater treatment facilities and other production scenarios. In nature, duckweed grows rapidly in stagnant ponds during the summer months. However, man-made duckweed ponds often are susceptible to algae growth and must be harvested frequently to maintain optimal biomass production.

Studying duckweed associated bacteria (DABs) may provide an important management tool for large-scale production of duckweed biomass reliably and sustainably. In general, plant growth promoting bacteria (PGPB) have been shown to improve plant health by increasing nutrient availability, providing defense against pathogens, protection from abiotic stresses, and producing phytohormones such as auxin (Santoyo et al., 2016). Recent studies have revealed that DABs can improve the growth of duckweed by increasing frond production rate as well as higher chlorophyll content (Yamaga et al., 2010; Suzuki et al., 2014). Some duckweed associated bacterial strains were found to breakdown phenol contaminants or increase the removal of nitrogen and phosphate from wastewater by these aquatic plants, which may be correlated with their ability to promote plant growth (Yamaga et al., 2010; Suzuki et al., 2014). After inoculation of a PGPB isolated from the surface of the duckweed, *Lemna aoukikusa* (indistinguishable from *Lemna aequinoctialis* using atpF-atpH and psbK-psbI barcodes Borisjuk et al., 2015), there was an observed increase in chlorophyll content in the monocotyledon *Lemna minor* as well as the dicotyledon *Lactuca sativa* (Suzuki et al., 2014). Thus understanding which microbes promote plant growth and the mechanisms they use to do so, could help establish a more sustainable approach of increasing duckweed biomass as well as the growth of other plants.

There is evidence that the plant phytohormone, auxin, may act as a signal molecule between bacteria and plants (Bianco et al., 2006; Lui and Nester, 2006; Spaepen et al., 2007). IAA is the most common auxin found in nature. It is suggested that over 80% of rhizosphere bacteria may be capable of synthesizing IAA (Spaepen and Vanderleyden, 2011). In certain cases, high concentrations of IAA production by bacteria may increase overall root biomass, allowing the plant to better uptake water and minerals, which in turn can enhance bacteria colonization (Spaepen and Vanderleyden, 2011). Indole is a less common, naturally occurring compound that has been shown to be a signal molecule between microbes in biofilm formation and quorum sensing (Martino et al., 2003; Lee and Lee, 2010). In contrast to the case of IAA, the role of various indole-related compounds during plant-microbe interactions is poorly understood. Therefore, our aim in this work was to focus on indole related compounds as an initial screen to identify plant growth promoting duckweed-associated bacteria (DABs). Future work may involve screening for effects on the production of other plant hormones such as gibberellin or ethylene by strains in our collection. In this work, we have characterized a set of 47 duckweed-associated bacteria (DABs) based on their production of indole related compounds by using the Salkowski reagent and mass spectrometry. The Salkowski reagent consists of ferric chloride and perchloric acid that when mixed with IAA produces a pink color (Gordon and Weber, 1951). Well characterized *Azospirillum brasilense* strains Sp7 and Sp245, originally isolated as plant-associated bacteria from wheat tissues (Bashan and de-Bashan, 2010), were used in our work as positive controls since they are known to produce IAA and one is an epiphyte (Sp7) while the other is an endophyte (Sp245) in wheat. Analysis of duckweed associated endophytes reveals that the production of indole is correlated with the DAB being associated with the *Wolffia* genus whereas the production of indole related compounds such as IAA is correlated with those DABs that were isolated from the *Lemna* genus. Indole and indole related compounds, IAA and indole-3-lactic acid (ILA), were detected from cultures of a subset of DABs in our collection and their potential role in improving duckweed plant health is discussed.

MATERIALS AND METHODS

Duckweed Tissue

Plant material was obtained from the Rutgers Duckweed Stock Cooperative at Rutgers University, New Brunswick, NJ, USA. Plant strains were maintained in 60 × 15 mm Petri dish on 0.5X Schenk and Hildebrandt (SH) Basal Salt Mixture supplemented with 0.8% w/v Difco Agar, granulated. Plants were additionally maintained on 0.5X SH medium with 0.8% w/v agar and 0.5% sucrose with and without the addition of 100 mg/L cefotaxime. The plants were grown at a temperature of 15°C under illumination of 40–44 μmol m^{-2} s^{-1} light. Duckweed strains stored at the Rutgers Duckweed Stock Cooperative originate from various locations in the world (Supplementary Table 1). Duckweed strains were also collected June 2015 from various locations in New Jersey, USA (Supplementary Table 1).

Isolation of Bacteria

To isolate duckweed associated bacterial strains, duckweed tissue was surface sterilized before placing on LB (Miller) agar medium or Tryptic Soy Agar (TSA). Duckweed tissues were rinsed in 1.5 mL eppendorf tubes with sterile Rinse Solution (0.1% Triton-X, 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4{\cdot}7H_20$, 1.8 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) for 1 min and then decanted. For isolation using bleach, an extra step was performed by washing the tissue in 0.6% (v/v) sodium hypochlorite until only the meristem retained chlorophyll and then washing with 2% $Na_2S_2O_3$ for 1 min to neutralize the bleach. The solution was decanted and the tissue was washed in sterile Millipore water for 1 min. The tissue was then homogenized using a plastic drill and then spread onto LB agar plates or TSA plates. The plates were incubated at 28°C for up to 72 h. Colonies on the plates were picked and re-streaked several times on LB or TSA plates for single colonies to obtain pure isolates. Bacterial strains were stored at −80°C in LB or Tryptic Soy Broth (TSB) supplemented with 40% sterilized glycerol.

Fluorescent Microscopy

To confirm that the bleach treatment that we employed to isolate candidate endophytic bacteria from duckweed can effectively remove most if not all surface-associated bacteria, fluorescent microcopy was performed on *Lemna minor* strain 370-DWC112 inoculated with DAB 1A (Supplementary Figure 1). Plant tissue was treated with 0.3% (v/v) sodium hypochlorite for 2 min using the method described for isolation of bacteria, which left only the meristem to retain chlorophyll. The tissue was placed in a microcentrifuge tube with 100 μL of 6 μM Syto 9 stain (ThermoFisher Scientific, Waltham, MA). The Syto 9 stain was removed after 3 min and the tissue was washed twice with 200 μL of sterile water. The tissue was then placed on a microscope slide and observed using an Olympus FSX100 epifluorescence microscope (460–495 nm excitation/510–550 nm emission). To image the surface of the plant tissue, the 10x objective lens was first used to focus on the guard cells of the stomates before switching to higher magnification objectives to resolve stained bacteria on the same focal plane.

Genotyping Duckweed Strains

Plant DNA was isolated by grinding 100 mg fresh weight of the tissue in liquid nitrogen and 500 μL of 1x CTAB buffer with 5 μL of 2-mercaptoethanol. The extract was incubated at 65°C for 30 min. 1x volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The extract was centrifuged at 10,000 × g for 5 min and then the aqueous phase was transferred into a new tube. Two microliter of RNase A (10 mg/mL) was added and the sample was incubated at room temperature for 30 min. The phenol/chloroform/isoamyl alcohol step was repeated one more time before adding 0.5x volume of 7.5 M ammonium acetate and 2.5x volume of 100% ethanol to the aqueous phase. The sample was centrifuged at 16,000 × g for 30 min at room temperature. The supernatant was decanted and 500 μL of 70% ethanol was added. The sample was centrifuged at 10,000 × g for 5 min and the supernatant was decanted. The pellet was air dried and then resuspended in 5 μL of sterile Millipore water. Concentration and quality of DNA was determined with a Nanodrop-1000 UV/Vis Spectrophotometer (Thermo Scientific, Waltham, MA) and then by running an aliquot of the sample on a 1% agarose gel with ethidium bromide followed by visualization on a transilluminator, respectively.

PCR amplification of the *atpH* and *psbK* regions of the plastid genome was performed to identify duckweed collected from the environment (Borisjuk et al., 2015). For the *atpH* gene the forward primer was ACTCGCACACACTCCCTTTCC and the reverse primer was GCTTTTATGGAAGCTTTAACAAT. For the *psbK* locus, the forward primer was TTAGCATTTGTTTGGCAAG and the reverse primer was AAAGTTTGAGAGTAAGCAT. One hundred nanogram of DNA was added to the amplification mixture containing 1x buffer, 0.25 mM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer, 4 mM $MgCl_2$, and 2 U of Taq polymerase. The PCR condition was Stage 1 at 95°C for 5 min, Stage 2 with 35 cycles at 95°C for 30 s, 51°C for 30 s, 72°C for 1 min, and Stage 3 at 72°C for 5 min.

Sequencing of 16S rRNA Gene

PCR amplification of the 16S rRNA gene was performed on single bacterial colonies using the bacterial universal primers e9f forward (GAGTTTGATCCTGGCTCAG) and e926r reverse (CCGTCAATTCCTTTGAGTTT) (Baker et al., 2003; Chakravorty et al., 2007). DNA was added to the amplification mixture containing 1x buffer, 0.2 mM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer and 2 U of Taq polymerase. The PCR condition was Stage 1 at 95°C for 5 min, Stage 2 with 25 cycles at 95°C for 1 min, 50°C for 30 s, 72°C for 1 min, and Stage 3 at 72°C for 5 min.

Sequence Analysis

Sanger sequencing of the *atpH*, *psbK,* and 16S rRNA genes was performed by Genewiz Co. (Piscataway, New Jersey) and sequences were analyzed using Serial Cloner and UGENE programs. Supplementary Table 1 lists the accession numbers for the 16S rRNA sequences registered in Genbank. With the support by the Joint Genome Institute (JGI)-Environmental Molecular Sciences Laboratory (EMSL) Collaborative Science program, the complete genome sequences for 33 of our DAB collection have been determined. Analysis of complete genome sequences was performed using JGI, KEGG Mapper, and RAST annotation service (Aziz et al., 2008; Overbeek et al., 2013; Brettin et al., 2015). Amino acid sequence alignment of tryptophanase and % identity matrix was performed using Clustal Omega version 1.2.4 with default parameters.

Colorimetric Detection of Indole Related Compounds

Bacterial strains from glycerol stocks were streaked onto either an LB agar plate or a TSA plate, depending on the medium of their original isolation and grown at 28°C. For each strain, a single colony was used to inoculate 6 mL of liquid LB medium, with and without 5 mM L-tryptophan. For DAB 33B and DAB 39B, liquid TSB (with and without 5 mM L-tryptophan) was used instead due to difficulty growing these two strains on LB medium. After 48 h of growing at 28°C with shaking at 240 rpm, 1 mL of culture was centrifuged for 5 min at 14,000 rpm to collect the supernatant. The original Salkowski assay based on the Gordon and Weber protocol was adapted for a 96-well format (Gordon and Weber, 1951). In a Corning 96-well clear bottom white plate, 100 μL of the supernatant was added to 200 μL of Salkowski reagent (10 mM $FeCl_3$, 97% reagent grade, and 34.3% perchloric acid, ACS grade) in duplicate. After incubating samples with the Salkowski reagent at room temperature for 30 min, the color change was recorded. A BioTek Synergy HT microplate reader was used to determine the absorbance (O.D.) at a single wavelength of 530 nm. To estimate the amount of indole related compounds at 530 nm, an IAA standard curve was generated by suspending IAA (Gibco Laboratories, Life Technologies, Inc., New York, USA) in 100% acetonitrile at a concentration of 1 mg/mL and diluting in LB medium or TSB to a concentration of 100, 50, 20, 10, 5, and 0 μg/mL. Sterile LB medium (with and without 5 mM L-tryptophan) and sterile TSB (with and without 5 mM L-tryptophan) were used as controls. The concentration of indole related compounds at 530 nm of

the sterile control sample, either LB or TSB depending on the bacterial medium used, was subtracted from the concentration of indole related compounds at 530 nm of the bacterial samples to obtain a background subtracted concentration.

A full spectrum analysis from 440 to 600 nm, using a 1 nm interval, was performed to identify the wavelength of maximum absorbance. Full spectrum analysis was performed on bacterial samples grown in liquid LB medium supplemented with 5 mM L-tryptophan whereas free indole and free IAA (as references) were suspended in 100% acetonitrile. The wavelength of maximum absorbance (λ_{max}) was calculated between 460 and 600 nm due to the high background signal observed at wavelengths shorter than 460 nm from addition of the Salkowski reagent to LB medium.

For determining the specificity of the Salkowski reagent, we tested IAA, indole-acetamide (IAM), indole-3-pyruvatic acid (IPA), ILA, indole-3-butyric acid (IBA), indole, indoxyl sulfate, tryptophol, and tryptophan. The compounds were suspended in 100% acetonitrile, HPLC grade, before diluting in LB medium, which did not contain 5 mM L-tryptophan due to the high absorbance background tryptophan generates when performing a spectrum analysis from 440 to 600 nm wavelength.

Extraction of Indole Related Compounds

For extraction of indole related compounds, bacterial strains from glycerol stocks were streaked onto an LB agar plate and grown at 28°C. A single colony was used to inoculate a starter culture of 6 mL liquid LB medium, supplemented with 5 mM L-tryptophan, and grown at 28°C and 240 rpm. After 24 h, the starter culture was used to make a 60 mL culture of liquid LB medium, supplemented with 5 mM L-tryptophan, at OD_{600} 0.01. The cultures were grown at 28°C and 240 rpm. After 24 h, the supernatant was collected by centrifugation at 10,000 × g for 5 min. For the spike sample, 300 μg of free IAA was added to 60 mL of LB medium supplemented with 5 mM L-tryptophan. All of the samples were then acidified with 1 N HCl to a pH of 2.7. The samples were then separated into 20 mL aliquots for biological triplicates. A Sep-Pak C18 cartridge (360 mg sorbent, 55–105 μm particle size) was prepared for each sample by washing with 10 mL of 100% acetonitrile followed by 10 mL of water. The acidified supernatant was passed through the C18 cartridge. The C18 cartridge was then washed with 10 mL of water and eluted with 5 mL of 80% (v/v) acetonitrile. The eluate was vacuum concentrated and then stored at−20°C until ready for LC/MS. Concentrated samples were dissolved in 1 mL of 100% acetonitrile. The samples were sonicated twice for 15 min and then centrifuged at 14,000 rpm for 5 min to remove any solid particles. A standard curve was generated by making IAA solutions of 0.5, 1, and 10 ng/μL in 100% acetonitrile. Acetonitrile of HPLC grade and HCl of ACS grade was used for the experiment and water was prepared from Millipore Synergy 185.

Detection of IAA by LC/MS

Samples were separated and analyzed by a UPLC/MS system including the Dionex® UltiMate 3000 RSLC ultra-high pressure liquid chromatography system, consisting of a workstation with ThermoFisher Scientific's Xcalibur v. 4.0 software package combined with Dionex®'s SII LC control software, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector the eluent flow was guided to a Q Exactive Plus Orbitrap high-resolution high-mass-accuracy mass spectrometer (MS). Mass detection was full MS scan with low energy collision induced dissociation (CID) from 100 to 1,000 m/z in either positive or negative ionization mode with electrospray (ESI) interface. Sheath gas flow rate was 30 arbitrary units, auxiliary gas flow rate was 7, and sweep gas flow rate was 1. The spray voltage was 3,500 volts (−3,500 for negative ESI) with a capillary temperature of 275°C. The mass resolution was 140,000 and the isolation window was 0.4 mDa. Substances were separated on a Phenomenex™ Kinetex C8 reverse phase column, size 100 × 2 mm, particle size 2.6 mm, pore size 100 Å. The mobile phase consisted of 2 components: Solvent A (0.5% ACS grade acetic acid in LCMS grade water, pH 3–3.5), and Solvent B (100% Acetonitrile, LCMS grade). The mobile phase flow was 0.20 ml/min, and a gradient mode was used for all analyses. The initial conditions of the gradient were 95% A and 5% B; for 30 min the proportion reaches 5% A and 95% B which was kept for the next 8 min, and during the following 4 min the ratio was brought to initial conditions. An 8 min equilibration interval was included between subsequent injections. The average pump pressure using these parameters was typically around 3,900 psi for the initial conditions.

Putative formulas of IAA metabolites and other indole related compounds were determined by performing isotope abundance analysis on the high-resolution mass spectral data with Xcalibur v. 4.0 software and reporting the best fitting empirical formula. Database searches were performed using reaxys.com (RELX Intellectual Properties SA) and SciFinder (American Chemical Society).

RESULTS

Isolation of Bacterial Endophytes

Duckweed tissues that were selected from the RDSC or collected from various locations in New Jersey, USA are of the genera *Wolffia, Lemna, Spirodela,* and *Landoltia* (Supplementary Table 1). Forty-seven strains of bacteria were isolated from 16 strains of surface sterilized duckweed tissue. Of the bacterial isolates, 62% belong to the Proteobacteria phylum, 23% belong to the Firmicutes phylum, 11% belong to the Actinobacteria phylum, and 4% belong to the Bacteroidetes phylum (Supplementary Table 1).

Specificity of the Salkowski Reagent

We tested the Salkowski reagent on various commercially available indole related compounds to determine the specificity of the assay (**Table 1**). IAA, IAM, and IPA, resulted in a color change to pink when these compounds were mixed with the Salkowski reagent. The wavelength which resulted in the maximum absorbance increase was 530 nm. IBA resulted in a color change to orange with a wavelength of maximum

TABLE 1 | Specificity of the Salkowski reagent was determined by testing indole related compounds.

Compound	Color change	Wavelength of maximum absorbance (nm)
Tryptophan	No color change	N/a
ILA	No color change	N/a
Tryptophol	No color change	N/a
IAA	Pink	530
IAM	Pink	530
IPA	Pink	530
IBA	Orange	450
Indole	Brown	490
Indoxyl sulfate	Purple	560

A color change indicates detection of one or more indole related compound(s). The wavelength recorded indicates the wavelength of maximum absorbance. ILA, Indole-3-lactic acid; IAA, Indole-3-acetic acid; IAM, Indole-acetamide; IPA, Indole-3-pyruvic acid; IBA, Indole-3-butyric acid. N/a indicates not applicable result.

absorbance increase at around 450 nm. Indoxyl sulfate resulted in a purple color change with a wavelength of maximum absorbance increase at around 560 nm and indole resulted in a brown color change with a wavelength of maximum absorbance increase at around 490 nm. Tryptophan, ILA and tryptophol resulted in no observable color change.

Colorimetric Detection of Indole Related Compounds

Using the Salkowski reagent as a colorimetric assay, we determined that 78.7% (37 out of 47) of the isolated DABs are capable of producing indole related compounds (Supplementary Table 2). Of those DABs producing indole related compounds, 81.1% are pink-type as observed by a color change from yellow to pink at a maximum absorbance increase at around 530 nm and 18.9% are brown-type as observed by a color change from yellow to brown at a maximum absorbance increase at around 480 nm (**Table 2** and Supplementary Table 2). As a positive control, we performed the Salkowski assay on *A. brasilense* strains Sp7 and Sp245, which are well-studied IAA producing PGPBs. When the Salkowski reagent was applied to the supernatant of Sp7 and Sp245, there was a color change to pink with an increase in absorbance that peaks at 530 nm. The concentration of indole related compounds in the sample was estimated using an IAA standard curve (Supplementary Table 3). The concentration of indole related compounds produced by pink-type bacteria, as compared to no color change bacteria, are listed in **Table 2**.

Synthesis of Indole Related Compounds Without Exogenous L-Tryptophan

A subset of 18 pink-type and 5 brown-type bacterial strains were further screened for their ability to synthesize indole related compounds without 5 mM L-tryptophan supplemented into the growth medium. The full absorption spectrum from 400 to 600 nm of an IAA standard curve from the Salkowski assay reveals that a wavelength of maximum absorbance (λ_{max}) cannot be detected at $\leq$5 μg/mL of IAA when no exogenous L-tryptophan is supplemented into the medium (Supplementary Figure 2). A λ_{max} cannot be detected at $\leq$10 μg/mL of IAA when 5 mM L-tryptophan is supplemented into the medium due to a high background absorbance. Four pink-type strains and one brown-type strain were able to synthesize indole related compounds without exogenous 5 mM L-tryptophan as determined by the color change after addition of the Salkowski reagent to the supernatant (**Figure 1**). This suggests that most of the DABs in our collection require an exogenous source of tryptophan to produce indole related compounds when cultured alone.

Using KEGG Mapper to analyze the complete genome sequences for 33 strains of our DAB collection, we identified tryptophan metabolism genes present in a subset of these bacterial genomes. Based on the estimated quantity of indole related compounds from the Salkowski assay, we compared the genomes of a pink-type top producer, a pink-type moderate producer and one that did not change color (DAB 37A, 1A, and 3D). We found that the pink-type top producer contained the most tryptophan metabolism-related genes of the three genomes compared. All three DABs contain a gene encoding a potential amidase enzyme, which may be able to convert IAM to IAA (Supplementary Figure 3 and Supplementary Table 4). Interestingly, the genome of DAB 3D which did not change color in the Salkowski assay, also appears to have this gene present, suggesting it may have the ability to convert IAM to IAA. However, based on the Salkowski assay, DAB 3D is not a producer of indole related compounds when grown alone in culture medium with or without addition of tryptophan.

Potential Correlation Between Production of Indole Related Compounds and Duckweed Genus

Because of the large proportion of pink-type DABs and an unexpected proportion of DABs that turned brown, we wanted to determine whether there was an association between the type of indole related compound produced by the DABs and the duckweed genus from which these DABs were isolated. We calculated that of the 30 pink-type DABs, 19 or 63.3% are derived from *Lemna* species and of the 6 brown-type DABs that were isolated from genotyped duckweed strains, 4 or 66.7% are derived from *Wolffia* species (Supplementary Table 2). Pearson's chi square test of independence was used to calculate the null hypothesis that production of indole related compounds from DABs is independent of the duckweed genus that the DAB was isolated from. The obtained chi square value from the 3 by 4 contingency table is 20.3 with a degree of freedom of 6 and a *p*-value of 0.002 (Supplementary Table 5). The *p*-value is less than the critical value of 0.05 and therefore we reject our null hypothesis.

To determine which categories of the 3 by 4 contingency table are associated, two 2 by 2 contingency tables were created. The first 2 by 2 contingency table was used to test the null hypothesis that pink-type DABs are independent of the duckweed genus *Lemna*. The obtained chi square value is 4.30 with a degree of

TABLE 2 | Estimation for the amount of indole related compounds detected by the Salkowski reagent in bacterial culture supernatants grown with 5 mM L-tryptophan.

A	**Pink-type**		
Bacteria genus	**Strain**	**ng/μL of indole related compound**	**Standard deviation**
	Sterile TSB	0	0.425
	Sterile LB	0	0.187
Pseudomonas	DAB D4EL2	3.485	0.973
Pseudomonas	DAB D2FKM1	3.666	1.918
Pseudomonas	DAB D2EKK1	4.376	0.125
Paenibacillus	DAB 26A	4.392	1.280
Rhodanobacter	DAB D4FL3	4.458	0.913
Microbacterium	DAB 19B	4.959	1.378
Paenibacillus	DAB 5M	5.036	1.929
Microbacterium	DAB 33B	5.182	2.873
Azospirillum	Sp7	5.396	3.024
Pseudacidovorax	DAB 35E	5.696	3.406
Paenibacillus	DAB 5A	6.967	1.586
Janthinobacterium	DAB D4EL3	7.484	3.200
Rhizobium	DAB 36D	7.533	2.690
Azosprillum	Sp245	7.764	1.333
Herbaspirillum	DAB 5O	8.424	3.206
Microbacterium	DAB 19A	8.564	2.154
Pseudomonas	DAB 36E	9.541	0.178
Rhizobium	DAB 35A	10.911	4.830
Herbaspirillum	DAB 5S	10.957	2.858
Paenibacillus	DAB 4T	11.196	2.501
Paenibacillus	DAB 4X	11.452	2.154
Paenibacillus	DAB 15A	11.719	0.818
Pseudomonas	DAB 38C	12.660	0.797
Herbaspirillum	DAB 5E	18.581	0.578
Bosea	DAB D4EK4	19.739	4.463
Microbacterium	DAB 1A	20.693	3.059
Microbacterium	DAB 1D	21.972	3.802
Acidovorax	DAB 35F	22.071	5.571
Azospirillum	DAB 38E	53.204	17.241
Rhizobium	DAB 20A	54.233	6.825
Rhizobium	DAB 33A	77.145	9.031
Azospirillum	DAB 37A	78.160	5.183
B	**No color change**		
Genus	**Strain**	**ng/μL of indole related compound**	**Standard deviation**
Bacillus	DAB 3D	0.586	0.459
Bacillus	DAB 27A	0.878	0.216
Staphylococcus	DAB 1B	0.746	0.273
Bacillus	DAB 2C	2.660	1.119
Bacillus	DAB 36A	1.868	0.198
Bacillus	DAB 36C	1.076	0.618
Pseudomonas	DAB 38D	1.521	0.524
Rhodanobacter	DAB D4EH1	2.016	0.729
Rhodanobacter	DAB D4FH1	0.729	0.792
Rhodanobacter	DAB D4EL1	1.125	0.374

Background subtracted concentrations were obtained by taking the average concentration as calculated from the standard curve and subtracting the concentration of either the sterile TSB or sterile LB sample, depending on the medium used to culture the bacteria. Means and standard deviation were calculated using 3 biological replicates. ***(A)*** *Mean concentration (ng/μL) of indole related compound and standard deviation of Sp7, Sp245, and pink-type DABs.* ***(B)*** *Mean concentration (ng/μL) of indole related compound and standard deviation of DABs resulting in no color change.*

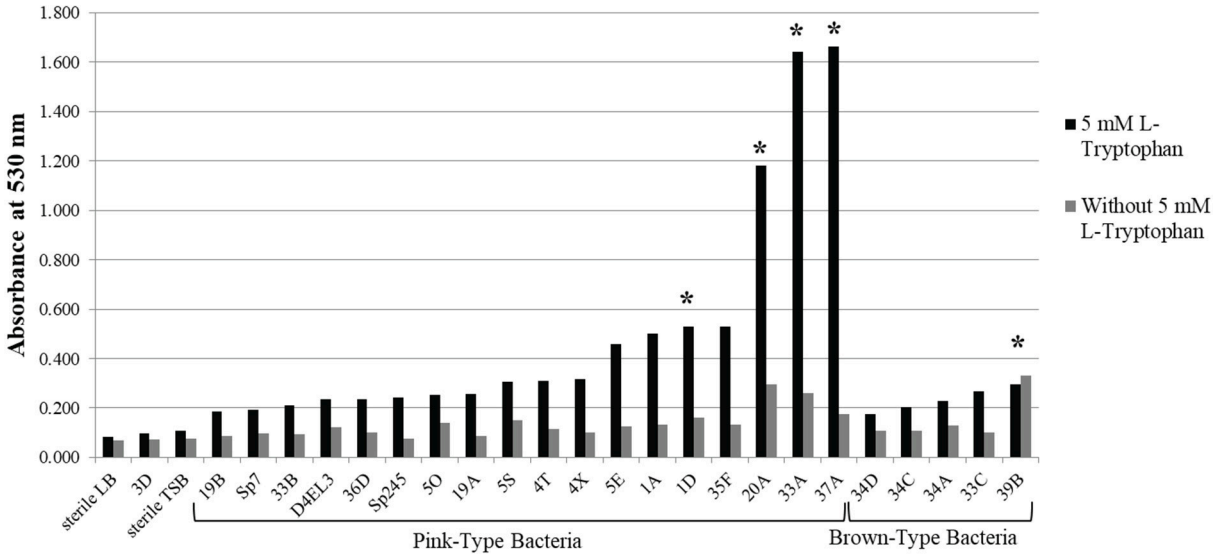

FIGURE 1 | Production of indole related compounds from duckweed associated bacteria strains. Addition of Salkowski reagent to the bacterial supernatant of a subset of the pink-type and brown-type bacteria strains grown in medium with and without 5 mM L-tryptophan to measure the absorbance of indole related compounds at 530 nm. *Indicates that the supernatant without 5 mM L-tryptophan resulted in an observable color change.

freedom of 1 and a *p*-value of 0.038 (Supplementary Table 5). The p value is less than the critical value of 0.05 and therefore we reject our null hypothesis. The second 2 by 2 contingency table was to test the null hypothesis that brown-type DABs are independent of the duckweed genus *Wolffia*. The obtained chi square value is 14.16 with a degree of freedom of 1 and a *p*-value of 0.0001 (Supplementary Table 5). The p value is much less than the critical value of 0.05 and therefore we reject our null hypothesis and conclude that there is a strong association between the brown-type DABs and the *Wolffia* genus, analogous to the association observed between the pink-type DABs and the *Lemna* genus. These results suggest that there is a significant correlation between the type of indole related compound produced by a particular DAB and the duckweed genus from which the DAB was isolated.

Identification of Indole Related Compounds by LC-MS

To more precisely validate the identity of the indole related compounds that are produced by the DABs, we first used LC-MS to determine whether the pink-type DABs were producing free IAA. The molecular weight of free IAA is 175 g/mol with positive ionization resulting in molecular ion at m/z 176 [M+H] and a fragment at m/z 130 (**Figure 2**). Negative ionization also resulted in molecular ion at m/z 174 [M–H] and a fragment at m/z 130. MS/MS was not performed due to the CID IAA fragmentation into an m/z 130 species representing the indole CH_2 bridge component of IAA. Furthermore, fragmentation into an m/z 130 species from a larger compound at a different retention time from that of IAA led us to identify indole-lactic-acid (ILA) in one of the bacterial samples, indicating that the method could resolve specific indole related compounds.

A solution of 5 ng/μL of free IAA in 100% acetonitrile was used to determine the retention time of free IAA in our LC-MS system, which was approximately 9.84 min (**Figure 3A**) in extracted single ion chromatogram. The extracted ion chromatogram peak areas of m/z 176 are shown in **Figures 3C–I** for pink-type strains (Sp7, DAB 1A, DAB 37A, and DAB 5E), brown-type strains (DAB 34D and 39B), and no color change strain DAB 3D. The extracted ion chromatograms demonstrate that the brown-type strains, DAB 34D, and DAB 39B, produce small but detectable amounts of free IAA compared to the higher amounts observed in the pink-type strains. The background signal from the species with an m/z value of 176 in the medium with a shorter retention time of 8.0–8.1 min is not observed in the Sp7 and DAB 37A samples since the amount of IAA is much higher in these cases and the scale of the Y-axis is for a larger range. A free IAA standard was used to determine the HPLC UV absorbance signal at 280 nm for quantification (Supplementary Figure 4). The UV 280 nm absorbance signal for quantification of free IAA produced by Sp7, DAB 1A, DAB 37A, and DAB 3D are shown in Supplementary Table 6. Using a spike sample of 5 μg/mL free IAA in LB medium added just before our extraction and concentration protocol, we calculated a 61.5% recovery from the extraction process (Supplementary Table 6). Estimation of IAA production based on the Salkowski assay method suggested that our *Azospirillum* strain, DAB 37A, was a top IAA producer. However, quantification using HPLC, which has better resolution and sensitivity for IAA detection, suggests that *Microbacterium* strain, DAB 1A, and control *Azospirillum* strain, Sp7, produce higher levels of free IAA.

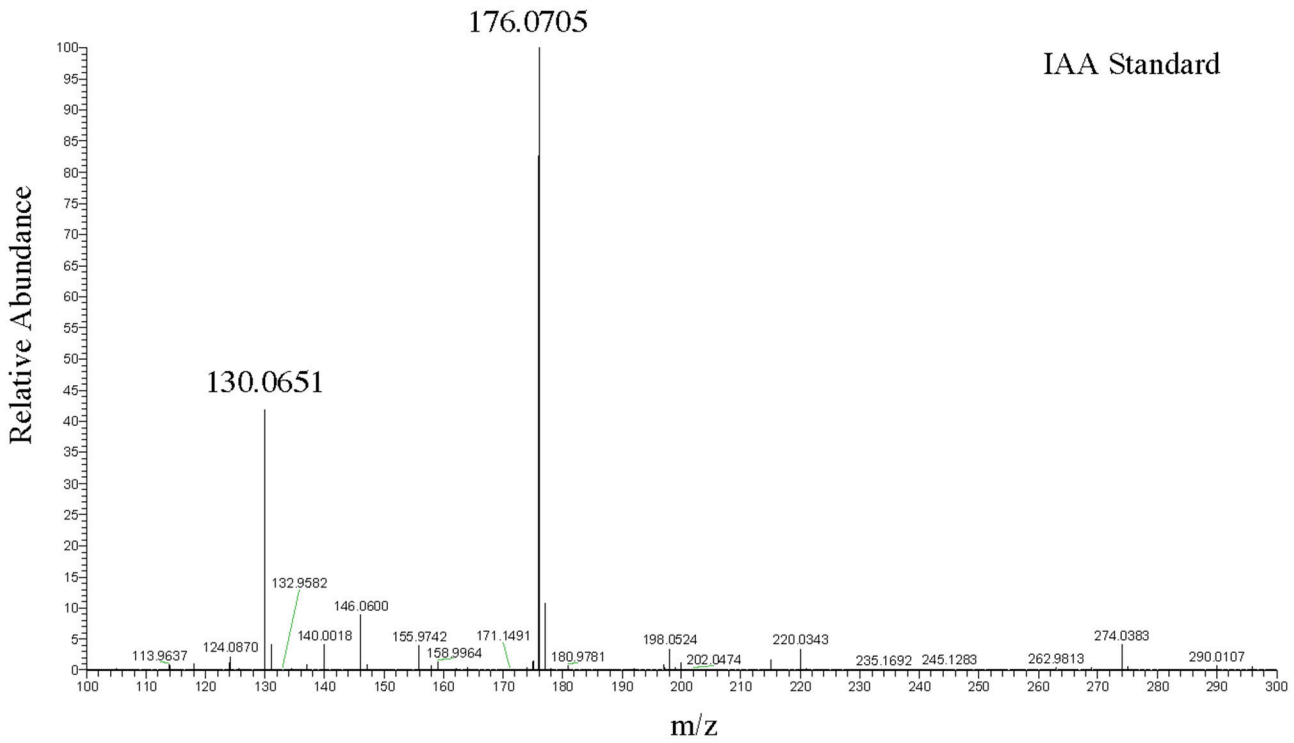

FIGURE 2 | LC-MS positive ion scan spectrum of free IAA. One hundred nanogram/microliter of free IAA in 100% acetonitrile at a retention time of 9.94 min.

Further LC-MS analysis revealed not only production of free IAA by the pink-type *Herbaspirillum* strain, DAB 5E, but also production of free ILA. This was detected by an m/z 130 fragment at a different retention time than IAA (Supplementary Figure 5). Free ILA did not result in a color change after addition of the Salkowski reagent (**Table 1**) and therefore, was only detectable using LC-MS. To verify the identity of the suspected ILA compound, a solution of 100 ng/μL of free ILA in 100% acetonitrile was used to determine the retention time of free ILA in our LC-MS system, which was approximately at 8.35 min (**Figure 4A**). Detection of free ILA with negative ionization resulted in a major peak at m/z 204 in extracted ion chromatogram. Extracted ion chromatograms at m/z 204 demonstrate that pink-type *Herbaspirillum* strain, DAB 5E produced free ILA in LB medium culture as well as a pink-type *Microbacterium* strain, DAB 1A, though at a much lower level (**Figures 4D,E**). These two DABs thus have an inverse ratio of IAA/ILA production with DAB 1A having a high IAA/ILA ratio while DAB 5E has a low IAA/ILA ratio (compare **Figures 3**, **4**).

Identification of the Major Salkowski-Positive Molecule in Brown-Type DABs as Indole

When using KEGG Mapper to identify tryptophan metabolism genes present in the DAB bacterial genomes, we noted the presence of a gene encoding tryptophanase in all of the brown-type DABs (Supplementary Figure 3), excluding DAB 34D which does not have the complete genome sequenced yet. This enzyme produces indole from tryptophan and this product could react in the Salkowski assay to produce a brown color that would be consistent with our observations. We BLASTed the amino acid sequence of the *E. coli* tryptophanase provided by KEGG Mapper (K-12 MG1655) to the amino acid sequence database of our DAB genomes. The sequence was 84% identical to the predicted homolog in the brown-type *Aeromonas* strain, DAB 39B, with an E value of 0.0 (Supplementary Figure 6 and Supplementary Table 7). The other brown-type DABs with genome sequence available also contain a predicted coding sequence that is similar to the *E. coli* typtophanase, although the percent identity was lower (Supplementary Figure 6 and Supplementary Table 7).

To determine whether the Salkowski assay result of a brown color change is due to indole, we performed the Salkowski assay on *E. coli*, strain DH5-alpha, which is a well-studied indole-producing bacterium. The result was a color change from yellow to brown with a wavelength of maximum absorbance increase at 490 nm (**Figure 5B**). When the Salkowski reagent was added to LB medium containing 1 mg/mL of free indole compound, the color change was from yellow to brown with a wavelength of absorbance maximum at 487 nm (**Figure 5D**). The full spectra from reaction with supernatants from brown-type *Aeromonas* strain, DAB 39B, brown-type *Chryseobacterium* strain, DAB 37D, and brown-type *Vogesella* strain, DAB 34D, are shown in **Figure 5** along with the full spectrum of LB medium containing both 1 mg/mL of free indole and 0.1 mg/mL of free IAA. There appear to be two peaks of absorbance corresponding to indole

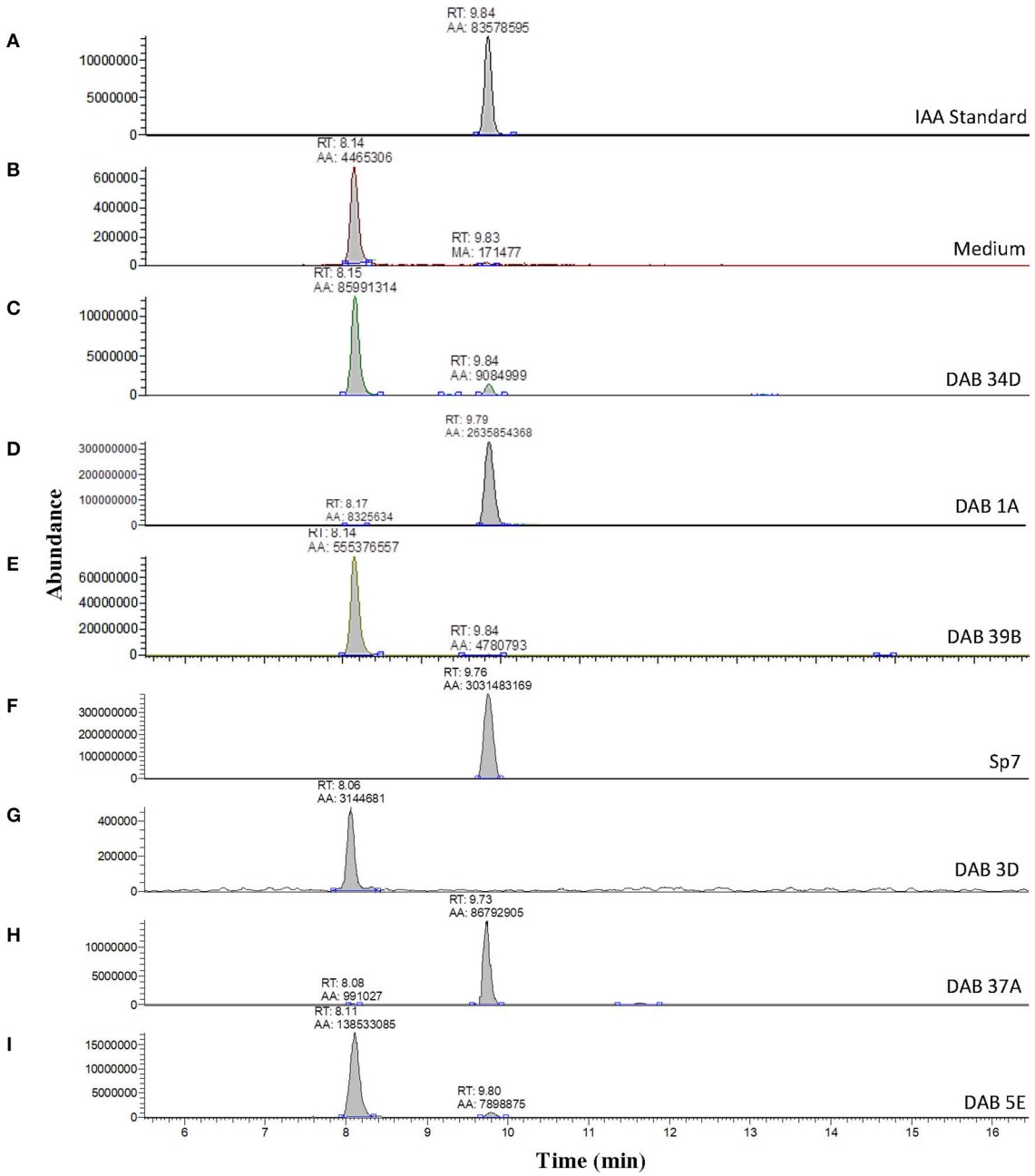

FIGURE 3 | Validation of IAA production in DABs by mass spectrometry. LC-MS extracted positive ionization ion chromatograms at m/z 176.06 to m/z 176.08 ([M+H] of IAA). RT, retention time; AA, automatic area under peak; MA, manual area under peak. **(A)** Chromatogram (EIC) from an injection of 5 ng/μL of free IAA suspended in 100% acetonitrile showing a retention time of 9.84 min. **(B)** EIC showing a low background signal at the RT of free IAA in the LB medium sample. A second signal is detected at a retention time of 8.14 min in the LB medium sample. **(C)** EIC from the LB medium with brown-type *Vogesella* strain, DAB 34D. **(D)** EIC from the LB medium with pink-type *Microbacterium* strain, DAB 1A. **(E)** EIC from the LB medium with brown-type *Aeromonas* strain, DAB 39B. **(F)** EIC from the LB medium with pink-type *Azospirillum* strain, Sp7. **(G)** EIC from the LB medium with no color change *Bacillus* strain, DAB 3D. **(H)** EIC from the LB medium with pink-type *Azospirillum* strain, DAB 37A. **(I)** EIC from the LB medium with pink-type *Herbaspirillum* strain, DAB 5E. The scales on the Y-axis are automatically adjusted to show all the peaks in each of the chromatograms. Note the different scale on the chromatogram in **(B)** to show the lower quantity peaks.

and IAA for DAB 39B, DAB 34D, and DAB 37D. Some of the DAB strains thus appear to be producing multiple indole related compounds during *in vitro* growth with potentially indole being one of them.

Extraction and Detection of Indole From DAB Strains by LC-MS

We used LC-MS to more precisely validate the identity of the suspected indole compound produced in the brown-type DABs.

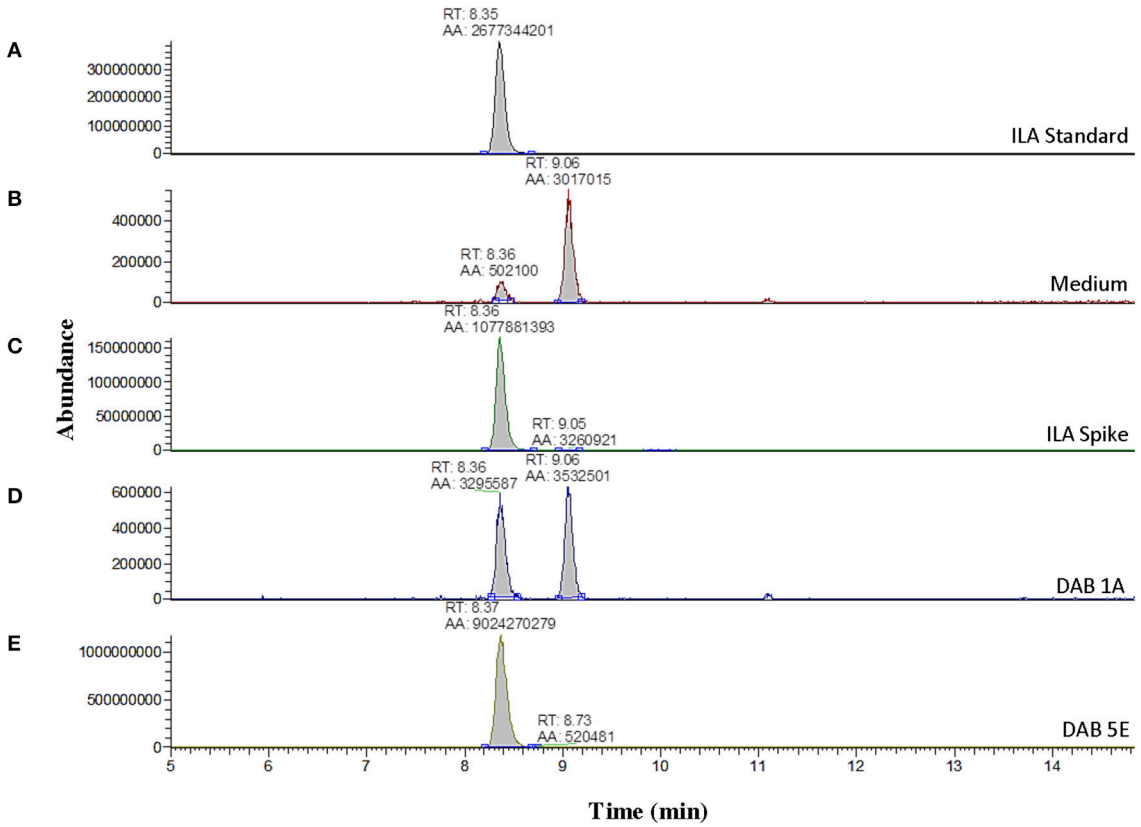

FIGURE 4 | Detection of indole-3-lactic acid production in two DABs by mass spectrometry. LC-MS extracted negative ionization ion chromatograms at m/z 204.06 to m/z 204.07 ([M-H] of ILA). RT, retention time; AA, automatic area under peak. **(A)** EIC of an injection of 100 ng/μL of free ILA suspended in 100% acetonitrile showing a retention time of 8.35 min. **(B)** EIC showing a background signal at the RT of free ILA in the LB medium sample. A second signal is detected at a retention time of 9.06 min in the LB medium. **(C)** EIC from the LB medium spiked with 100 ng/μL of ILA. **(D)** EIC from the LB medium with pink-type *Microbacterium* strain, DAB 1A. **(E)** EIC from the LB medium with pink-type *Herbaspirillum* strain, DAB 5E. The scales on the Y-axis are automatically adjusted to show all the peaks in each of the chromatograms. Note the different scale on the chromatograms in **(B,D)** to show the lower quantity peaks.

A solution of 100 ng/μL of free indole in 100% acetonitrile was used to determine the retention time of free indole in our LC-MS system, which was approximately 13.30 min (**Figure 6**). Sample preparation and extraction of free indole were performed identically to the extraction of free IAA. Detection of free indole with positive ionization resulted in a major peak at m/z 118 in extracted ion chromatogram. Using the peak area at m/z 118 of the samples, we detected free indole present in the supernatants of the brown-type *Vogesella* strain, DAB 34D, and the brown-type *Aeromonas* strain, DAB 39B (**Figure 6**). No indole was detected with supernatant from the pink-type *Microbacterium* strain, DAB 1A. A peak corresponding to IAA was also detected in the supernatants of brown-type strains DAB 34D and DAB 39B, but at much lower levels compared to the pink-type strain, DAB 1A (**Figure 3**). Our results thus provided strong evidence for the identity of indole as the major compound produced by the brown-type DABs and not produced by the pink-type DABs.

DISCUSSION

The Salkowski colorimetric assay is traditionally used to detect the bacterial production of IAA. However, after testing various indole related compounds, IAM and IPA also resulted in a color change to pink with an absorbance maximum at 530 nm. Similar findings were previously reported in which IAA, IPA, and IAM all reacted with the Salkowski reagent to cause a pink color change, however sulfuric acid was used rather than hydrochloric acid (Glickmann and Dessaux, 1995). We used hydrochloric acid based on a previous report that it results in greater color intensity than sulfuric acid and thus could be more sensitive (Gordon and Weber, 1951). Our present study, along with a previous report, indicates that the Salkowski assay alone is not sufficient to distinguish which indole related compounds are produced by bacteria (Glickmann and Dessaux, 1995). This is also evident by the mass spec detection of ILA produced by pink-type *Herbaspirillum* strain, DAB 5E, which could not have been detected by the Salkowski reagent since it does not react with this particular indole related compound to produce a visible color change. Studies using the Salkowski assay should thus be cautious of these limitations and employ additional techniques such as mass spectrometry for specific detection and positive identification of the indole related compounds.

The isolated endophytic DABs were primarily from the phyla Proteobacteria, Firmicutes, and Actinobacteria, which has

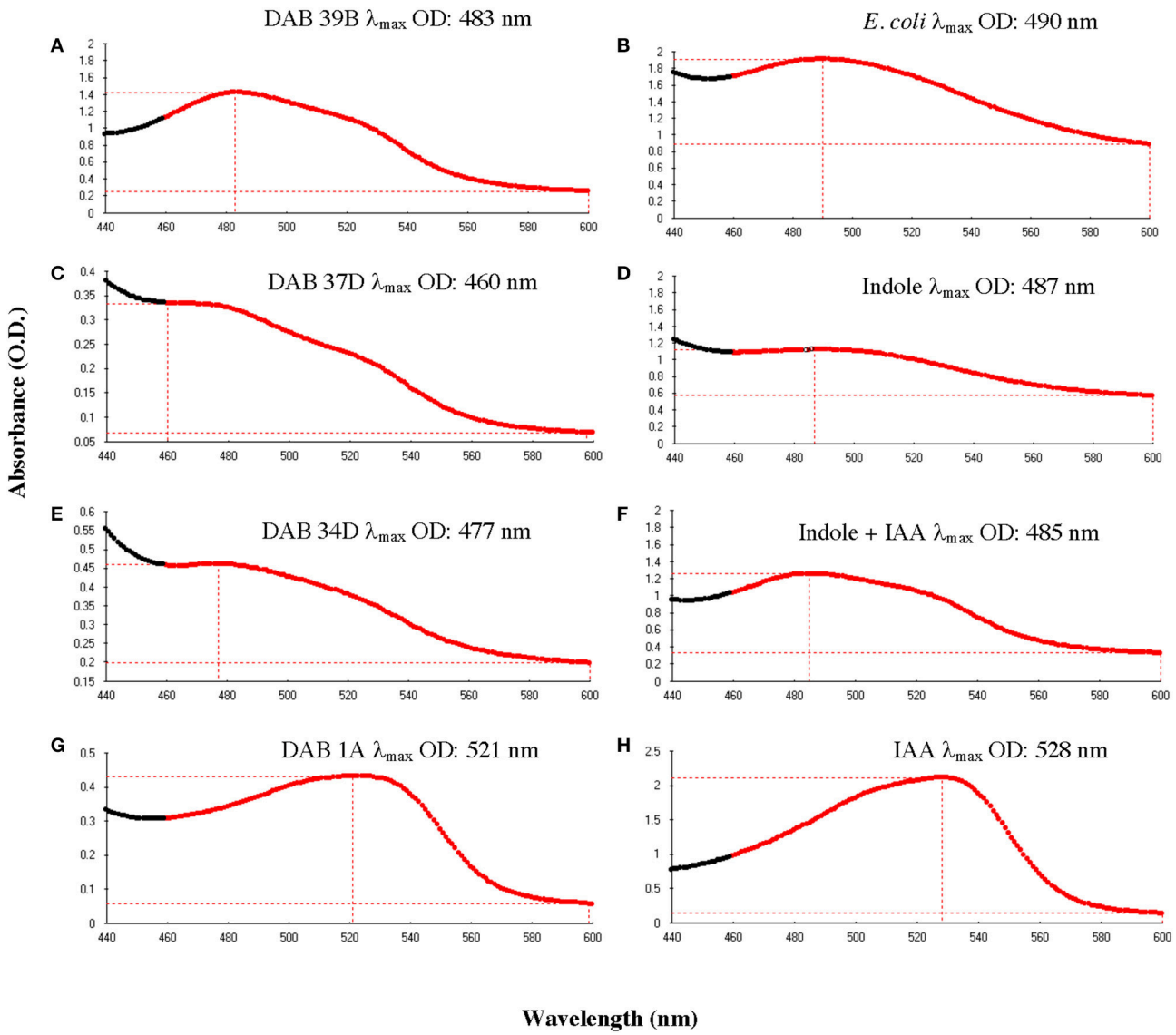

FIGURE 5 | Comparison of absorbance spectra between DABs and other reference samples with the Salkowski assay. Absorbance spectrums from 440 to 600 nm to determine the wavelength at the maximum OD (λ_{max}). **(A)** Brown-type *Aeromonas* strain, DAB 39B, has a λ_{max} at 483 nm and a minor peak at around 530 nm. **(B)** Known indole-producing *E. coli* strain, DH5 alpha, has a λ_{max} at 490 nm. **(C)** Brown-type *Chryseobacterium* strain, DAB 37D, has a λ_{max} at 460 nm and a minor peak at around 530 nm. **(D)** An LB solution containing 1 mg/mL of free indole has a λ_{max} at 487 nm. **(E)** Brown-type *Vogesella* strain, DAB 34D, has a λ_{max} at 477 nm and a potential minor peak at around 530 nm. **(F)** An LB solution containing a mixture of 1 mg/mL of free indole and 0.1 mg/mL of free IAA has a λ_{max} at 485 nm. **(G)** Pink-type *Microbacterium* strain, DAB 1A, has a λ_{max} at 521 nm. **(H)** An LB solution containing 0.1 mg/mL of free IAA has a λ_{max} at 528 nm.

been similarly reported for the land plant, *Arabidopsis thaliana* (Bulgarelli et al., 2012; Lundberg et al., 2012). This indicates that the core microbiome of duckweeds may be quite similar to those of land plants such as the dicot *A. thaliana* and highlights the conservation of plant-microbe association mechanisms as far back as the divergence between monocots and dicots about 150 million years ago (Chaw et al., 2004). Of the 47 endophytic DABs used for the Salkowski assay in this study, 79% were capable of producing indole related compounds. This is similar to the suggested 80% of epiphytic bacteria reported in literature capable of producing IAA (Spaepen and Vanderleyden, 2011). However, the result in a brown color change after addition of the Salkowski reagent has not been widely reported. Mass spectrometry confirmed the presence of free IAA in the pink-type DABs and the presence of free indole in addition to free IAA in the brown-type DABs, although the levels of IAA in the latter cases are substantially lower. In addition, our genome analysis of non-Salkowski-reactive *Bacillus* strain, DAB 3D, revealed the presence of a potential amidase gene, which may convert IAM to IAA. However, the Salkowski assay and mass spectrometry did not detect the presence of free IAA in the DAB 3D supernatant. One explanation could be that the gene is not expressed when

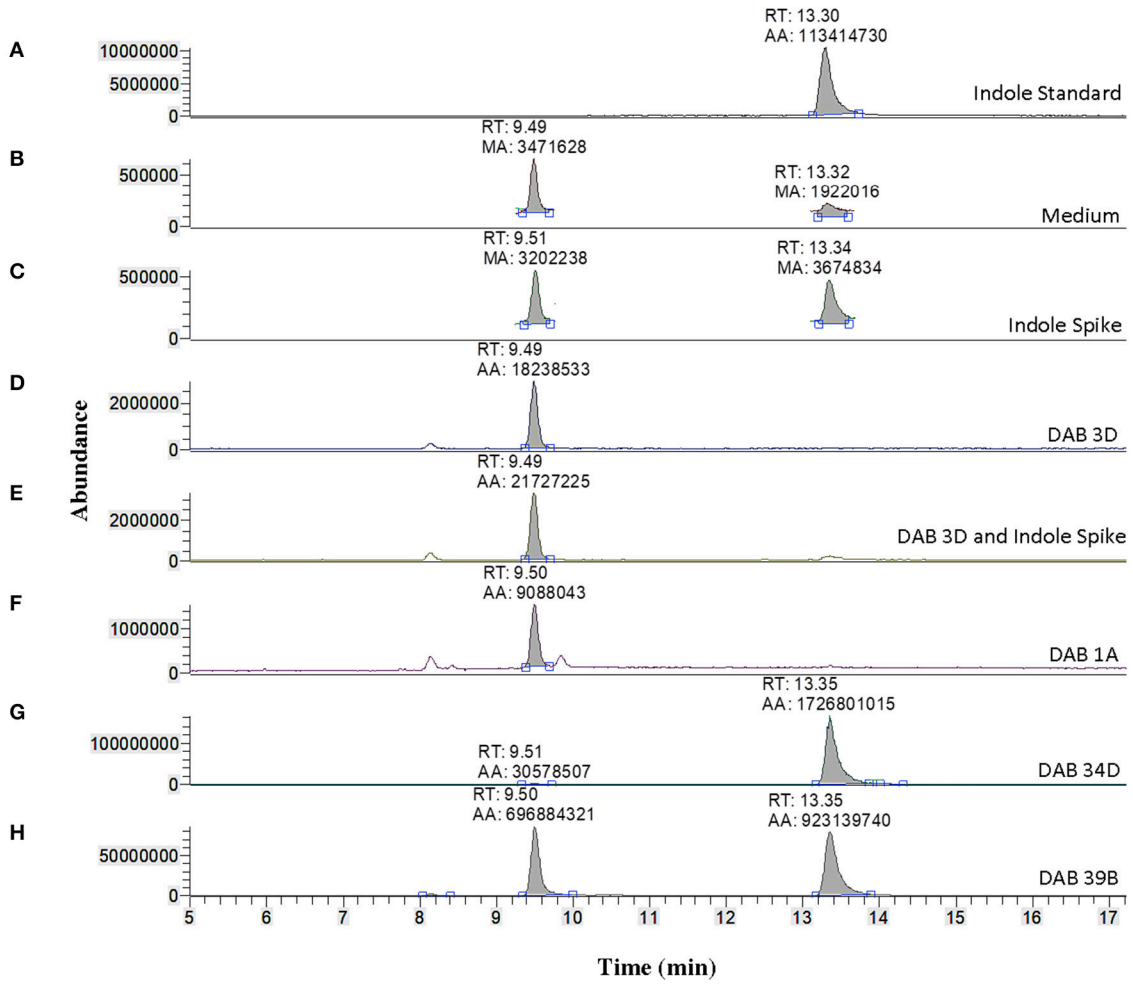

FIGURE 6 | Identification of indole production in brown-type DABs by mass spectrometry. LC-MS extracted positive ionization ion chromatograms at m/z 118.06 to m/z 118.07 ([M+H] of indole). RT, retention time; AA, automatic area under peak; MA, manual area under peak. **(A)** EIC from an injection of 100 ng/μL free indole in 100% acetonitrile showing a retention time of 13.30 min. **(B)** EIC showing a low background signal at the RT of free indole in the LB medium sample. A second signal is detected at a retention time of 9.49 min in the LB medium. **(C)** EIC showing free indole in the LB medium spiked with 100 ng/μL of free indole. **(D)** EIC from the LB medium with non-color *Bacillus* strain, DAB 3D. **(E)** EIC from the LB medium with DAB 3D spiked with 100 ng/μL free indole. The indole peak area is <LoQ at the scale of the chromatogram. **(F)** EIC from the LB medium with pink-type *Microbacterium* strain DAB 1A, showing no detectable indole. **(G)** EIC from the LB medium with brown-type *Vogesella* strain DAB 34D, showing a clear indole signal. **(H)** EIC from the LB medium with brown-type *Aeromonas* strain DAB 39B, showing a clear indole signal. The scales on the Y-axis are automatically adjusted to show all the peaks in each of the chromatograms. Note the different scale on the chromatogram in **(B,C)** to show the lower quantity peaks.

the bacteria is grown as a monoculture *in vitro*. Whether this is a pseudogene or if it is tightly regulated in DAB 3D to respond to specific cues remains to be determined. Future work is thus needed to determine whether some DABs are capable of producing free IAA when interacting with the duckweed hosts or in a community context.

In this study, we have also found that exogenous tryptophan is necessary for 79% of the tested DABs to produce indole related compounds during *in vitro* growth. The majority of the DABs may thus require exogenous tryptophan supplied by the plant to produce indole related compounds or they may have to be induced by the plant host to produce more tryptophan themselves. A previous study supports this notion that tryptophan-like compounds secreted by the plant root may stimulate IAA synthesis of PGPBs (Kamilova et al., 2006). Regardless of the cause for this requirement, our observation suggests that the DAB's phytohormone production capability during *in vitro* growth per se does not necessarily result in phenotypic effects of heightened auxin levels when inoculated onto the plant. Future experiments studying interactions of these DABs with various duckweed genera and environments will be necessary to better understand the dynamic control for this trait in different context of plant-microbe interaction. Our present results thus serve as a necessary foundation to better interpret future studies comparing the phenotypic output of different combinations of DAB and plant hosts. In this context,

the availability of whole genome sequences for both DABs and high quality reference genomes for duckweed such as *Spirodela polyrhiza* (Michael et al., 2017) should enable the application of molecular tools to begin to characterize the signaling pathways involved.

The need to more closely examine mechanisms used by endophytic DABs to associate with the plant is further supported by the finding of an apparent correlation between the production of specific indole related compounds and the duckweed genus that the DAB was isolated from. Specifically, pink-type DABs are more likely to be associated with DABs isolated from *Lemna* species whereas brown-type DABs are overrepresented in DABs that were isolated from *Wolffia*. Enrichment of specific bacteria phyla by different plant host genotypes has been previously reported (Lundberg et al., 2012; Edwards et al., 2015; Haney et al., 2015). Our present work indicates one additional selection factor could be the particular type of genes for indole related compounds that the bacteria may be able to synthesize. The effect of IAA on plant root development has been well characterized and more recently studies have demonstrated that it could function as a signal molecule between bacteria in a community as well as between bacteria and the plant host (Spaepen and Vanderleyden, 2011). On the other hand, indole production by bacteria has been shown to be involved in a number of microbial processes including quorum sensing and biofilm formation (Lee and Lee, 2010). However its effect on plant health and development is poorly understood compared to that of IAA. *Wolffia* plants are morphologically distinct from *Lemna* species in that they are usually comprised of a more spherical frond and are rootless. Thus, architecturally, colonization of *Wolffia* plants by DABs may pose different challenges and different strategies/mechanisms, such as formation of a biofilm, could be needed in the bacteria. One possible explanation for our findings is that either the duckweed or the endophytic bacteria is selecting for a symbiont in which the appropriate resources it requires, whether it may be a specific indole related compound or a particular plant morphology, could be utilized and enable colonization of the microbe on the plant host. More studies will be necessary to uncover the role of indole producing DABs on the health of different duckweed genera and to delineate the function for the indole that could be produced by these bacteria.

Finally, we believe that understanding a potential coevolution between duckweeds and their associated endophytes is critical for selecting the appropriate combination of bacteria when designing synthetic bacterial communities. We may utilize bacteria to target specific or multiple duckweed species and therefore, it will be useful to understand how the different duckweed genera interact with various bacterial strains in order to distinguish general and species-specific rules and pathways. This will be important for researchers trying to improve large scale duckweed farming in open, non-sterile conditions for wastewater treatment, biofuel or bioplastic production, and animal feed supplement, which currently remains important challenges to overcome.

AUTHOR CONTRIBUTIONS

EL conceived the experiments. EL, JX, KA, SL, and SG performed isolation and genotyping of bacterial strains and duckweed strains. SG performed the Salkowski test and genome analysis. SG prepared extraction of auxin related compounds for mass spectrometry. AP performed mass spectrometry. SG wrote the paper. KA, SL, AP, and EL provided input and revisions to 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/fchem.2018.00265/full#supplementary-material

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Nutritional Value of the Duckweed Species of the Genus *Wolffia* (Lemnaceae) as Human Food

Klaus-J. Appenroth[1], K. Sowjanya Sree[2], Manuela Bog[3], Josef Ecker[4], Claudine Seeliger[4], Volker Böhm[5,6], Stefan Lorkowski[5,6], Katrin Sommer[7], Walter Vetter[7], Karla Tolzin-Banasch[8], Rita Kirmse[8], Matthias Leiterer[8], Christine Dawczynski[5,6], Gerhard Liebisch[9] and Gerhard Jahreis[5,6]*

[1] Matthias Schleiden Institute, Plant Physiology, Friedrich Schiller University Jena, Jena, Germany, [2] Department of Environmental Science, Central University of Kerala, Kasaragod, India, [3] Institute of Botany and Landscape Ecology, University of Greifswald, Greifswald, Germany, [4] Chair of Nutrition Physiology, Technical University Munich, Freising, Germany, [5] Institute of Nutritional Sciences, Friedrich Schiller University Jena, Jena, Germany, [6] Competence Cluster for Nutrition and Cardiovascular Health (nutriCARD) Halle-Jena-Leipzig, Jena, Germany, [7] Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany, [8] Thuringian State Institute of Agriculture, Jena, Germany, [9] Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Regensburg, Germany

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Jitendra Paul Khurana, University of Delhi, India

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***Correspondence:**
Klaus-J. Appenroth
klaus.appenroth@uni-jena.de

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Species of the genus *Wolffia* are traditionally used as human food in some of the Asian countries. Therefore, all 11 species of this genus, identified by molecular barcoding, were investigated for ingredients relevant to human nutrition. The total protein content varied between 20 and 30% of the freeze-dry weight, the starch content between 10 and 20%, the fat content between 1 and 5%, and the fiber content was ~25%. The essential amino acid content was higher or close to the requirements of preschool-aged children according to standards of the World Health Organization. The fat content was low, but the fraction of polyunsaturated fatty acids was above 60% of total fat and the content of n-3 polyunsaturated fatty acids was higher than that of n-6 polyunsaturated fatty acids in most species. The content of macro- and microelements (minerals) not only depended on the cultivation conditions but also on the genetic background of the species. This holds true also for the content of tocopherols, several carotenoids and phytosterols in different species and even intraspecific, clonal differences were detected in *Wolffia globosa* and *Wolffia arrhiza*. Thus, the selection of suitable clones for further applications is important. Due to the very fast growth and the highest yield in most of the nutrients, *Wolffia microscopica* has a high potential for practical applications in human nutrition.

Keywords: amino acids, duckweed, fatty acids, Lemnaceae, phytosterols, protein, *Wolffia*

INTRODUCTION

Duckweeds represent a small family of aquatic floating monocots consisting of 37 species distributed all over the world (Landolt, 1986; Sree et al., 2016). These plants are the fastest growing angiosperms (Sree et al., 2015; Ziegler et al., 2015) and may cover ponds or lakes within a few days under favorable growth conditions. It is frequently observed that animals, such as ducks, swans, or geese, feed on duckweeds growing naturally in ponds or lakes. Of course, this is where the name, duckweed, comes from. These plants have also been used for a long time to feed domesticated

animals, either by providing them temporary access to duckweed grown ponds or by supplementing their diet with harvested duckweed, fresh or dried; in case of pigs, it has been already reported in the 1960s, and later reports with cattle, rams, sheep, horses, waterfowls, and fishes have been detailed by Landolt (1986). The World Bank had also supported a project to feed fish with duckweeds in Bangladesh (Skillicorn et al., 1993). More recently, detailed reports were published in this regard, e.g., using *Wolffia arrhiza* meal as a substitute for soya in the diet of Japanese quails (Suppadit et al., 2012), using duckweed species in the feed of striped catfish (Da et al., 2013), of rohu and carp (Sharma et al., 2016), of broilers (Shammout and Zakaria, 2015), and use of genetically modified *Lemna minor* to feed laying hens (Ghosh et al., 2015).

Duckweeds, in several Asian countries, also serve as human food. With the local names of *khai nam*, *kai-pum,* or *kai nhae* (literally meaning: water-eggs) the rootless duckweed *Wolffia globosa* is sold in the vegetable markets in different regions of Thailand. Bhanthumnavin and McGarry (1971) and Rusoff et al. (1980) investigated some of the duckweed species and suggested them as a possible source of protein. In these countries, fresh *Wolffia* plants are used to prepare several dishes like salads, omelets or vegetable curries (Saengthongpinit, 2017). Protein quantity and quality are important features especially in those countries with starch-rich staple food like rice and maize (Appenroth et al., 2017). In a recent publication, we provided an overview of the nutritional status of the whole plant family, Lemnaceae, and investigated species from each of the five genera concerning their protein content, amino acid spectrum, starch content, fat content, and fatty acid distribution (Appenroth et al., 2017). Moreover, we selected the fastest growing species, *Wolffia microscopica* to investigate the mineral composition, phytosterols and fiber content. From the set of species selected for the study, *W. microscopica* and *Wolffiella hyalina* were the most promising candidates with respect to their nutritional value (Appenroth et al., 2017). *W. microscopica* is an endemic species to the Indian subcontinent and *W. hyalina* has a closed distribution pattern. However, some of the other species of the genus *Wolffia* have a widespread distribution. *W. globosa* is a species mostly used for human nutrition in the Asian countries. Unlike the species belonging to the subfamily Lemnoideae (i.e., *Spirodela*, *Landoltia*, and *Lemna*), species of the genus *Wolffia* have the advantage that the oxalate content is not present in the form of calcium oxalate crystals that might cause health problems to humans (Landolt and Kandeler, 1987). In our previous paper, where it has been demonstrated that some of the duckweed species have excellent qualities for human nutrition, these species were selected as representatives of the duckweed genera. In the present paper, we addressed the question whether different species of the same genus contain similar or varying nutritional qualities concerning human nutrition by investigating all the 11 existing species of the genus *Wolffia* with a prime focus on *W. globosa*.

Abbreviations: AA, amino acids; Ala, alanine; ALA, α-linolenic acid; Arg, arginine; Asp, aspartic acid; Cys, cysteine; DW, dry weight; EAA, essential amino acids; FA, fatty acids; FAO, Food and Agriculture Organization of the United Nations; FAME, fatty acid methyl esters; FDW, freeze-dry weight; GLA, gamma-linolenic acid; Glu, glutamic acid; Gly, glycine; Ile, isoleucine; LA, linoleic acid; LCFA, long-chain fatty acids; Leu, leucine; LDL, Low density lipoprotein; Lys, lysine; Met, methionine; MUFA, monounsaturated fatty acids; Phe, phenylalanine; PUFA, polyunsaturated fatty acids; SCFA, short-chain fatty acids; SD, standard deviation; SDA, stearidonic acid (C18:4c6,9,12,15); SFA, saturated fatty acids; Thr, threonine; Trp, tryptophan; Val, valine; WHO, World Health Organization.

MATERIALS AND METHODS

Plant Material And Cultivation

Plant material was taken from the collection of duckweed strains, or clones, of the Department of Plant Physiology, University of Jena, Germany. The duckweeds in this collection, most of which stem from the collection of Prof. Elias Landolt, ETH, Zurich, Switzerland, were maintained under axenic conditions as described before (Appenroth et al., 1996). All 11 species of the genus *Wolffia* were represented by one clone each. The species *W. globosa* and *W. arrhiza*, however, were additionally represented by four and three clones, respectively (**Table 1**).

Determination of *Wolffia* species on morphological basis alone is very difficult and sometimes not reliable (Landolt, 1994). Hence, the identity of each clone was confirmed by barcoding using several plastidic sequences (Bog et al., 2013; **Supplementary Table S1**) as suggested by Borisjuk et al. (2015). The plants were pre-cultivated and cultivated as described before (Appenroth et al., 2017), except that the modified Schenk-Hildebrandt medium was replaced by a modified Steinberg medium with increased Fe^{3+} and EDTA concentrations as this medium turned out to guarantee more stable cultivation of *Wolffia* plants with fewer infections in the 15 L-plastic trays. The composition of this medium was as follows (Appenroth, 2015): KNO_3 3.46 mM, KH_2PO_4 0.66 mM, K_2HPO_4 72 μM, $MgSO_4$

TABLE 1 | List of all 11 species of the genus *Wolffia* used in the present investigations.

Genus	Species	Clone	Origin	Doubling time (d)
Wolffia	*angusta*	8878	Malaysia	1.3 ± 0.2
Wolffia	*arrhiza*	8618	Kenya	1.6 ± 0.1
		8853	Brazil	2.3 ± 0.0
		9528	Germany	1.8 ± 0.0
Wolffia	*australiana*	7540	New Zealand	1.4 ± 0.0
Wolffia	*borealis*	9123	USA	1.6 ± 0.0
Wolffia	*brasiliensis*	7925	Argentina	1.4 ± 0.1
Wolffia	*columbiana*	7155	USA	2.3 ± 0.1
Wolffia	*cylindracea*	9056	Zimbabwe	2.2 ± 0.1
Wolffia	*elongata*	9188	Colombia	1.6 ± 0.0
Wolffia	*globosa*	5514	Thailand	2.0 ± 0.0
		5515	Thailand	2.2 ± 0.1
		5537	Thailand	1.8 ± 0.2
		9498	India	1.2 ± 0.0
Wolffia	*microscopica*	2005	India	1.0 ± 0.0
Wolffia	*neglecta*	9149	Pakistan	1.2 ± 0.1

Beside clone identity number, origin of the clones and growth rates, given as doubling time ± SD (for definition cf. Ziegler et al., 2015), are presented. d, days.

0.41 mM, $Ca(NO_3)_2$ 1.25 mM, H_3BO_3 1.94 µM, $ZnSO_4$ 0.63 µM, Na_2MoO_4 0.18 µM, $MnCl_2$ 0.91 µM, Fe(III)NaEDTA 14.05 µM, EDTA-Na_2 6.1 µM, pH was adjusted to 5.5. Because of the higher requirement of fresh weight for all analyses, the cultivation time was extended to 2–3 weeks.

Growth rates of all plants were determined and weekly yield (using the parameter fresh weight, FW) was calculated based on the exponential law defined as yield after seven days of cultivation starting with 1 g FW as described by Ziegler et al. (2015).

Analytical Methods

After freeze-drying of fresh duckweeds, the freeze-dry weight (FDW) was determined. The freeze-dried material was finely ground and homogenized with a laboratory mill and aliquoted for further analyses. All analyses were carried out using the same freeze-dried material and the data were related to FDW.

The analyses of dry matter, ash, total fat, and total protein were carried out as described before (Appenroth et al., 2017). Further components were analyzed as described below.

Starch content: The starch content was measured following acidic extraction and Lugol's staining (Appenroth et al., 2010).

Total fiber content: The content of total fiber was enzymatically determined according to the supplier's protocol (α-amylase, protease, amyloglucosidase; BIO-QUANT, Total Dietary Fiber, Merck).

Amino acids: Amino acids (AA) were determined after subjecting the samples to acid hydrolysis with phenolic hydrochloric acid. For the sulfur-containing amino acids, methionine and cysteine, an oxidation step was performed before acid hydrolysis. The analysis of AA was carried out by means of ion exchange chromatography (Biochrom 30 Plus, Laborservice Onken, Gründau, Germany) and post column derivatization with ninhydrin.

Fatty acids (FA): Analyses were performed as described previously (Ecker et al., 2012). Briefly, fatty acid methyl esters (FAMEs) were generated by acetyl chloride and methanol treatment and extracted with *n*-hexane. Total FA analysis was generally carried out using a Shimadzu 2010 GC/MS system. FAMEs were separated on a BPX70 column (10-m length, 0.10-mm internal diameter, 0.20-µm film thickness) from SGE using helium as carrier gas. The initial oven temperature was 50°C and was programmed to increase at 40°C/min to 155°C, 6°C/min to 210°C, and finally 15°C/min to 250°C. Two different methods were used to analyze FAMEs to achieve maximum analytical coverage. The FA species and their positional and *cis/trans* isomers were characterized in scan mode and quantified by single ion monitoring to detect specific fragments of saturated and unsaturated FAs (saturated, *m/z* 74; monounsaturated, *m/z* 55; diunsaturated, *m/z* 67; polyunsaturated, *m/z* 79). The internal standard was non-naturally-occurring C21:0 *iso* without stearidonic acid. For the determination of steridonic acid an additional run was carried out. FAMEs were separated using gas chromatography (GC-17A with flame ionization detector, Shimadzu, Japan) and a medium polar column (DB-225MS, Agilent Technologies, USA). The standard FAME mixture for this run contained identical stearidonic acid (SDA; C18:4-c6,9,12,15). To calculate the fatty acid percentage, the concentration of each detected FAME species was divided through the total concentration of FAMEs before multiplication with 100, i.e., ([c]FAME species/ [c]total FAME)*100.

Minerals: After microwave pressure acid digestion, macro elements and most of the trace elements were determined by inductively coupled plasma atomic emission spectroscopy (iCAP 6000, Thermo Fisher Scientific). For digestion 0.2 g of the ground, freeze-dried material were weighed in a quartz vessel and 2 ml of ultrapure water and 5 ml of 65% HNO_3 were added. The vessels were closed and heated in the microwave digestion system (turboWAVE, Milestone S.r.L.) for 25 min (600–1,200 W). The digestion solution was filled up to 15 ml with ultrapure water. For the quantitative measurement of arsenic and selenium, the method of hydride generation atomic absorption spectrometry (PinAAcle 500, PerkinElmer) was used. The mercury content was determined from the solid matter by direct mercury analyzer (DMA-80, Milestone S.r.L.). For the analysis of iodine, a strong alkaline digestion of the freeze-dried material with aqueous solution of tetramethylammonium hydroxide (TMAH) came to use (0.5 g/5 ml ultrapure H_2O/ml 25% TMAH). Measurement was carried out by inductively coupled plasma mass spectroscopy (NexION 350X, PerkinElmer). All quantifications were performed as independent duplicate analysis. The relative standard deviation (RSD) for all parameters was determined from independent repeat measurements of the control sample over a period of several months.

Antioxidants: Carotenoids and tocopherols were extracted and determined as recently described (Appenroth et al., 2017).

Phytosterols: Sterols were extracted and determined, in duplicate, using GC/MS according to Appenroth et al. (2017) with the following modifications. The silylated unsaponifiable matter was analyzed with a Thermo Scientific Trace GC/Trace DSQ MS system equipped with a split/splitless injector and a CTC PAL auto sampler. Sample injection (1 µL) was performed in splitless mode at 250°C. The initial carrier gas flow was set to 1 mL/min. After 0.1 min, a steep ramp of 500 mL/min/min was used to obtain a flow of 10 mL/min which was held for 1 min. Subsequently, the flow was reduced at 5 mL/min/min to 4 mL/min (hold time 2 min) and then at 1 mL/min/min to 1 mL/min which was maintained throughout the run. An HP5-MS UI column (30-m length × 0.25-mm internal diameter, 0.25-µm film thickness, Agilent Technologies) was used in combination with the following GC oven program. After 1 min at 55°C, temperature was raised at 25°C/min to 255°C, then at 1.5°C/min to 283°C and finally at 15°C/min to 300°C which was held for 8 min. Temperature of the transfer line was set to 280°C. Data were recorded from *m/z* 50 to 650 after a solvent delay of 7 min. Sterol separation was obtained by temperature program, whereas carrier gas flow program was used for the simultaneous determination of phytol and dihydrophytol. Total sterol content was calculated by internal standard calibration via 5α-cholestane. For the quantification of the phytols an external calibration curve was used.

Statistics

Lyophilized plant material from a single batch was used for all investigations. All data based on two independent measurements

($n = 2$). As reference systems, either the freeze-dry weight (content of protein, fat, fiber, minerals, carotenoids, tocopherol), or the total protein content (amino acids), or the total fat content (fatty acids, sterols) were used. The RSD gives the matrix-specific precision (repeatability) of the analytical procedure in percent under run-to-run conditions determined by long-term experiments (Ecker et al., 2012). Averages ± standard deviations (SD) are given for each amino acid, fatty acid, minerals, tocopherol, carotenoids, and phytosterols of all 16 *Wolffia* clones.

RESULTS

Overview of Nutritional Values of Species And Clones

In the freeze-dried material (about 92% dry weight, e.g., about 8% remaining water) of the eleven species of the genus *Wolffia*, the contents of protein, fat, starch, and fiber were determined (**Figure 1**). Because of the frequency of distribution and their traditional practical use in human nutrition, *W. globosa* and *W. arrhiza* were represented by four and three clones, respectively.

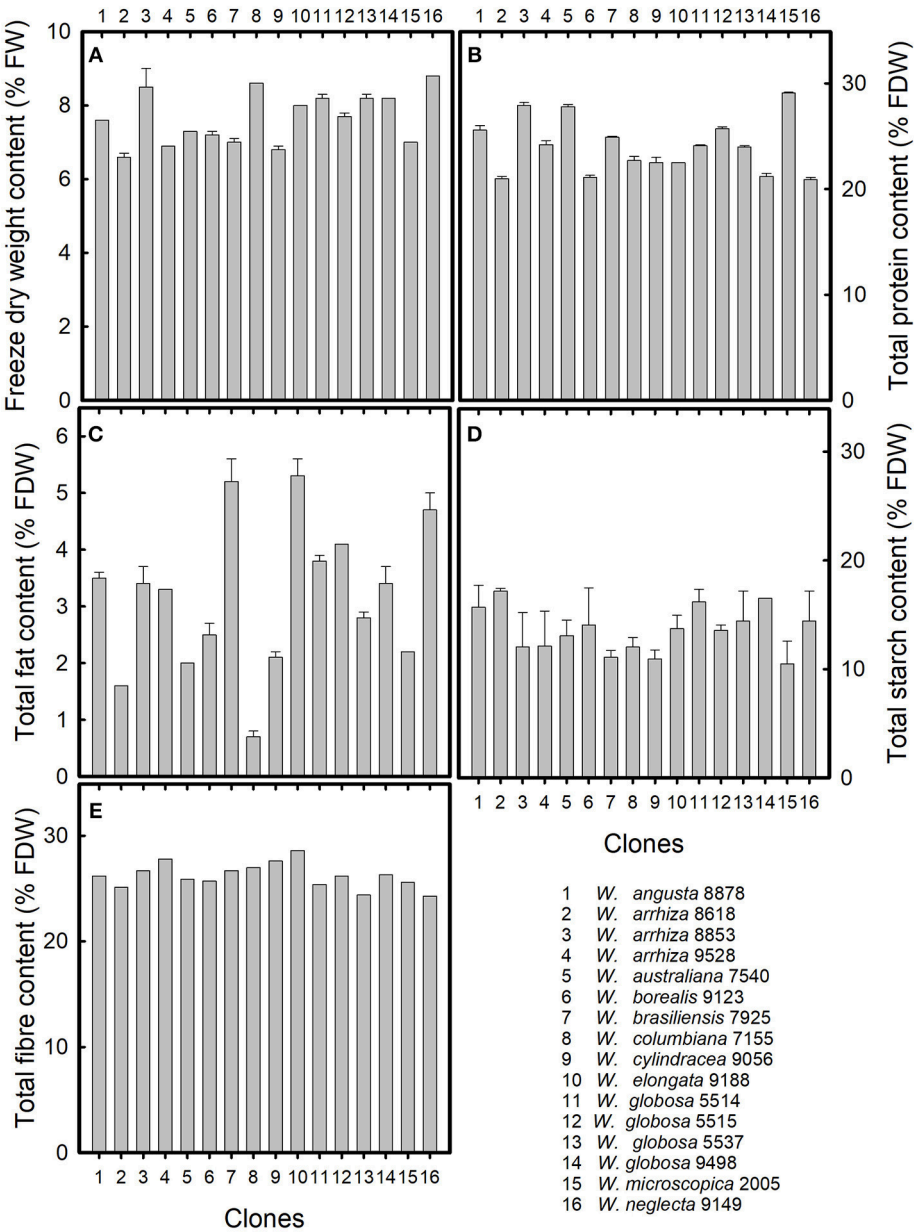

FIGURE 1 | Chemical composition of 11 species of the genus *Wolffia*. **(A)** Freeze dry weight in relation to fresh weight, and **(B)** total protein content, **(C)** total fat content, **(D)** total starch content, and **(E)** total fiber content in relation to freeze dry weight. Data were given as means together with standard deviations of parallel measurements. The numbers on the x-axis represent the species investigated. For further explanations, see **Table 1**.

TABLE 2 | Amino acid composition of protein of *Wolffia* species (g/100 g protein) (description of clones, **Table 1**).

Clone	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
W. angusta 8878	7.1	6.0	13.3	1.7	12.1	5.2	1.7	3.9	8.4	6.0	1.7	5.0	4.5	5.3	4.4	3.2	5.0
W. arrhiza 8618	6.7	5.9	9.6	1.3	11.7	5.2	1.9	3.8	8.3	6.0	1.6	5.2	4.6	4.8	4.0	3.3	5.0
W. arrhiza 8853	8.8	5.8	16.3	1.5	11.0	5.2	1.7	3.3	7.2	5.4	1.4	4.4	4.2	5.4	3.8	3.1	4.4
W. arrhiza 9528	8.8	5.4	13.0	1.4	10.8	5.2	1.7	3.3	7.1	4.9	1.4	4.3	4.0	5.4	3.6	2.7	4.5
W. australiana 7540	6.3	5.5	15.5	1.4	11.1	5.0	1.8	3.6	7.7	5.6	1.5	4.8	4.0	4.6	4.1	3.3	4.7
W. borealis 9123	6.6	5.8	10.4	1.4	11.4	5.3	1.9	3.8	8.0	6.0	1.7	5.0	4.7	4.6	4.2	3.5	5.1
W. brasiliensis 7925	6.6	6.4	13.5	1.6	12.9	5.0	1.9	3.7	8.1	6.0	1.5	4.9	4.3	4.7	4.0	3.5	5.1
W. columbiana 7155	6.7	5.3	11.4	1.7	11.8	5.7	1.6	3.2	7.2	5.2	1.3	4.5	3.8	5.5	3.4	2.8	4.3
W. cylindracea 9056	6.4	5.7	9.3	1.6	11.0	5.3	1.7	3.6	7.7	5.9	1.7	4.7	4.3	5.0	3.9	2.8	4.8
W. elongata 9188	6.6	6.2	9.6	1.4	11.2	5.4	2.2	3.9	8.6	5.7	1.7	5.3	4.8	5.2	4.6	3.6	5.1
W. globosa 5514	6.4	5.8	12.1	1.6	11.0	5.1	1.8	3.7	7.7	5.4	1.7	4.9	4.6	4.5	4.5	3.5	4.8
W. globosa 5515	6.5	5.8	14.0	1.5	10.8	4.7	1.6	3.3	6.9	5.0	1.5	4.2	4.0	4.2	3.9	3.1	4.3
W. globosa 5537	6.9	5.6	9.7	1.5	11.1	5.2	1.7	3.6	7.5	5.4	1.6	4.6	4.4	4.5	4.2	3.3	4.6
W. globosa 9498	6.4	5.8	10.8	1.6	11.0	5.5	2.0	3.8	7.7	5.6	1.5	4.7	4.6	5.0	4.2	3.4	4.9
W. microscopica 2005	6.5	5.8	12.8	1.6	12.2	5.2	1.8	3.8	8.1	6.0	1.7	4.8	4.3	5.0	4.5	3.2	5.0
W. neglecta 9149	6.4	5.9	10.6	1.6	11.4	5.6	1.9	3.7	7.9	6.1	1.7	4.7	4.6	5.7	4.3	3.4	5.1
RSD (%)	1.7	1.2	1.5	3.4	2.9	1.6	2.4	2.9	2.2	2.5	3.7	3.8	4.3	2.9	3.1	3.0	1.6
Average	6.9	5.8	12.0	1.5	11.4	5.2	1.8	3.6	7.8	5.6	1.6	4.7	4.4	5.0	4.1	3.2	4.8
± SD	0.8	0.3	2.2	0.1	0.6	0.2	0.2	0.2	0.5	0.4	0.1	0.3	0.3	0.4	0.3	0.3	0.3

Relative standard deviation (RSD) gives the matrix-specific accuracy (repeatability) of the analytical procedure in percent. Mean and standard deviation (SD) characterize the average content and variation of each analyzed amino acid across all 16 clones.

The freeze dry weight of the freshly harvested plant material was between ∼7 and 9% of the fresh weight (**Figure 1A**), the starch content was between 10 and 15% of FDW (**Figure 1D**), and the fiber content was about 25% of FDW (**Figure 1E**). Larger variations between the plant samples were detected for the protein content, varying between 20 and 30% (**Figure 1B**). The largest differences were found for total fat content (**Figure 1C**), varying between 0.7% in *W. columbiana* and 5.3% in *W. elongata*.

Amino Acid Distribution

The AA content of all investigated samples for 17 AA is given in **Table 2**. Especially for human nutrition, cysteine + methionine, threonine, phenylalanine, tyrosine, lysine, and leucine are important. Therefore, we compared the contents of critical AA with the reference pattern based on the essential amino acid requirements of the preschool-age child as published in 1985 [WHO (World Health Organization), 1985]. **Figure 2** reports the ratio between the measured AA content of the investigated clones (averages) with those of the reference (for details see **Supplementary Table S2**). In all samples, this ratio for isoleucine, leucine, cysteine + methionine, threonine, and valine was above 1. Lysine and the aromatic AA histidine + phenylalanine (H+F) were slightly limiting AA in duckweeds. Most of the ratios for lysine were close to 1 (*W. angusta* 8878, *W. arrhiza* 8618, *W. borealis* 9123, *W. brasiliensis* 7925, *W. cylindracea* 9056, *W. microscopica* 2005, and *W. neglecta* 9149; see **Supplementary Table S2**). In some cases, the ratio was slightly lower than 1, with the lowest value of 0.84 for *W. arrhiza* 9528. For the aromatic AA, histidine and phenylalanine, four of the duckweed clones had ratios slightly below 1 (between 0.92 and 0.97). The average of AA showed that practically all duckweed clones fulfilled the requirement for human nutrition (**Figure 2**). Several species had ratios above 1 compared to the reference protein.

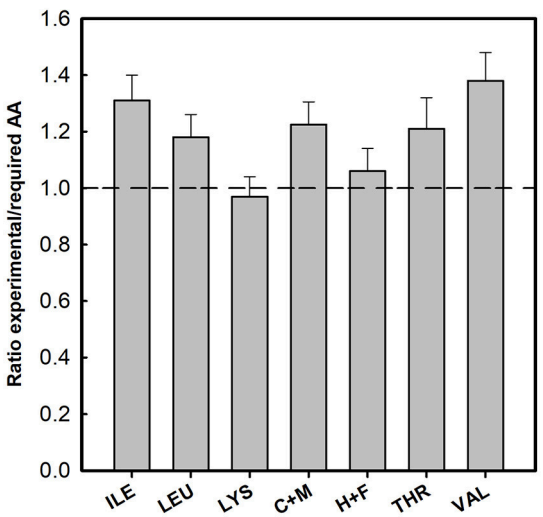

FIGURE 2 | Ratio between the essential amino acid content in duckweed clones and requirements in preschool-age children (WHO (World Health Organization), 1985). C+M, cysteine + methionine; H+F, histidine + phenylalanine. Data were given as average of all investigated clones (± SD), the data for the single *Wolffia* clones were given in the Supplementary Material (**Table S2**).

Fatty Acid Distribution

The fat in duckweed contained ∼34% saturated fatty acids with particularly high levels of palmitic acid (C16:0). Capric acid (C10:0), myristic acid (C14:0), margaric acid (C17:0), stearic acid (C18:0) and long-chain (>C18:0) saturated fatty acids (SFA) were present in rather small amounts or only in traces (**Table 3**). The highest fraction of SFA (**Tables 3**, **5**) was detected in *W. globosa* 9498 (∼42%), the lowest was in *W. arrhiza* 9528 (∼29%).

The monounsaturated fatty acids (MUFA), oleic acid (C18:1-c9), *cis*-vaccenic acid (C18:1-c11), and gondoic acid (C20:1-c11), were present (∼2 – 4%) in smaller amounts than SFA (**Tables 4**, **5**). *Wolffia elongata* 9188 comprised the highest MUFA fraction with 7.4%.

Most importantly (**Table 5**), the FA profile was dominated by polyunsaturated fatty acids (PUFA) with ∼54% in *W. globosa* 9498 and ∼68% in *W. arrhiza* 9528 (average across all *Wolffia* species: ∼63%). The major PUFA was α-linolenic acid (ALA, C18:3-c9,12,15; n-3 fatty acid), followed by linoleic acid (LA, C18:2-c9,12; n-6 fatty acid). γ-Linolenic acid (GLA; C18:3-c6,9,12; n-6 fatty acid) and C20:2-c11,14 (11,14-eicosadienoic acid) showed very low abundance (**Table 4**). The amount of ALA was always higher than the sum of LA and GLA. Therefore, the important n-6/n-3 PUFA ratio was consistently <1, ranging from 0.48 in *W. australiana* 7540 to 0.94 in *W. elongata* 9188 and *W. globosa* 9498 (**Table 5**). This was demonstrated by partially large RSDs of the averages (**Tables 3–5**). Stearidonic acid (SDA; n-3 PUFA) was solely detected in *W. microscopica* 2005 and in *W. australiana* 7540 (**Figure 3**) but was below the detection limit in all other clones.

Minerals

The total ash content amounted to ∼18% of FDW. The macro elements calcium, potassium, sodium, magnesium, iron and phosphorous (**Table 6**) as well as the microelements and non-essential heavy metals (**Tables 7**, **8**) mercury, arsenic, selenium, copper, manganese, zinc, iodine, and lead were measured in all 16 duckweed samples. As the mineral content can be easily adjusted to the specific requirements of human nutrition by changing the composition of the nutrient medium (Appenroth et al., 2017), we focused here on the enrichment of minerals in different plants under identical growth medium conditions. Magnesium content ranged from 1.91 to 4.55 g/kg FDW (mean 2.85 ± 0.71 g/kg FDW), iron from 0.11 to 0.4 g/kg FDW (mean 0.23 ± 0.09 g/kg FDW), and the trace element manganese from 78.4 to 431 mg/kg FDW (mean 230 ± 98 mg/kg FDW). Interestingly, iodine ranged from 0.20 to 0.92 mg/kg FDW (mean 0.39 ± 0.19 mg/kg FDW), and cadmium content from 0.009 to 0.59 mg/kg FDW (mean 0.076 ± 0.145 mg/kg FDW), although both elements were not applied by purpose and must have been introduced as impurities of chemicals or water. It should be stated here that *pro analysi* chemicals and purified and desalted water (<0.1 μS/cm) was used for growth medium preparation. Consequently, the uptake of minerals and trace elements also depends on the plant species studied.

Carotenoids And Tocopherols

In all 16 duckweed samples the contents of (*all-E*)-*β*-carotene, (*9Z*)-β-carotene, (*13Z*)-β-carotene, (*all-E*)-lutein, (*all-E*)-zeaxanthin, and α-tocopherol were analyzed (**Table 9**). The dominating carotenoid in all cases was clearly lutein (ca. 40–80

TABLE 3 | Distribution of saturated fatty acids in lipids of *Wolffia* species [% of FAME].

Clone	C10:0	C14:0	C16:0	C17:0	C18:0	C20:0	C22:0	C24:0	C26:0	C28:0
W. angusta 8878	0.13	0.33	30.0	0.29	2.2	0.68	0.36	0.63	0.55	0.24
W. arrhiza 8618	0.26	0.40	27.7	0.22	2.0	0.69	0.71	0.38	0.37	0.26
W. arrhiza 8853	0.12	0.34	30.8	0.23	1.8	0.42	0.55	0.39	0.39	0.32
W. arrhiza 9528	0.21	0.47	24.6	0.20	1.7	0.65	0.66	0.38	0.32	0.17
W. australiana 7540	0.20	0.41	28.4	0.19	2.2	0.62	0.85	1.50	0.44	0.13
W. borealis 9123	0.14	0.31	25.6	0.18	2.0	0.45	0.27	0.40	0.38	0.15
W. brasiliensis 7925	0.18	0.31	32.3	0.24	1.9	0.85	0.59	0.84	0.24	0.06
W. columbiana 7155	0.19	0.23	26.6	0.16	2.0	0.58	0.62	0.62	0.33	0.07
W. cylindracea 9056	0.18	0.44	29.9	0.23	1.9	0.60	0.67	0.43	0.35	0.22
W. elongata 9188	0.15	0.57	27.6	0.25	2.2	0.33	0.60	0.34	0.52	0.28
W. globosa 5514	0.20	0.30	30.0	0.22	2.3	0.40	0.24	0.43	0.40	0.17
W. globosa 5515	0.17	0.28	27.8	0.21	2.6	0.45	0.26	0.46	0.38	0.14
W. globosa 5537	0.17	0.41	28.1	0.20	2.1	0.43	0.26	0.49	0.40	0.14
W. globosa 9498	0.34	0.55	34.3	0.35	3.6	0.75	0.64	0.54	0.59	0.34
W. microscopica 2005	0.14	0.59	26.8	0.13	1.8	0.47	0.00	0.05	1.18	0.00
W. neglecta 9149	0.23	0.37	28.0	0.24	2.6	0.45	0.29	0.31	0.36	0.19
RSD (%)	10,2	4,4	6,2	5,5	5,8	9,4	7,5	6,5	9,5	9,5
Average	0.19	0.39	28.7	0.22	2.2	0.55	0.47	0.51	0.45	0.18
± SD	0.06	0.11	2.5	0.05	0.5	0.15	0.23	0.31	0.21	0.08

For further explanations see ***Table 2****.*

TABLE 4 | Distribution of mono- and polyunsaturated fatty acids in lipids of *Wolffia species* [% of FAME].

Clone	C18:1-c9 n-9	C18:1-c11 n-7	C18:2-c9,12 n-6	γC18:3-c6,9,12 n-6	αC18:3-c9,12,15 n-3	C20:1-c11 n-9	C20:2-c11,14 n-6
W. angusta 8878	3.47	0.23	20.5	n.d.	40.1	0.16	0.08
W. arrhiza 8618	2.54	0.42	25.9	n.d.	37.8	0.06	0.10
W. arrhiza 8853	2.58	0.51	28.7	n.d.	32.5	0.16	0.13
W. arrhiza 9528	2.00	0.59	24.6	n.d.	42.9	0.17	0.16
W. australiana 7540	1.91	0.80	19.1	1.01	42.0	0.08	0.10
W. borealis 9123	2.73	0.83	26.0	0.07	40.2	0.15	0.13
W. brasiliensis 7925	1.52	0.53	23.3	0.47	36.6	0.02	0.06
W. columbiana 7155	1.62	0.43	23.0	0.75	42.4	0.16	0.16
W. cylindracea 9056	2.76	0.53	24.9	n.d.	36.6	0.17	0.08
W. elongata 9188	6.18	1.03	27.5	1.38	30.7	0.16	0.09
W. globosa 5514	2.63	1.33	24.1	0.03	37.0	0.13	0.10
W. globosa 5515	2.95	0.78	25.9	0.03	37.4	0.16	0.10
W. globosa 5537	3.07	0.89	26.0	0.34	36.8	0.15	0.10
W. globosa 9498	2.55	0.80	26.1	0.22	28.1	0.13	0.09
W. microscopica 2005	1.20	0.50	26.2	1.60	37.0	0.09	0.09
W. neglecta 9149	2.76	0.75	27.6	0.21	35.4	0.14	0.11
RSD (%)	5,8	3,8	6,1	4,2	5,5	8,1	6,6
Average	2.65	0.68	25.0	0.38	37.1	0.13	0.10
± SD	1.12	0.27	2.5	0.53	4.1	0.04	0.03

n.d., not detectable. The limit of detection (LOD) was calculated as three times the noise signal of a blank. For further explanations see ***Table 2****.*

TABLE 5 | Fatty acid groups and n-6/n-3 ratio in lipids of *Wolffia* species [% of FAME].

Clone	Sum SFA	Sum MUFA	Sum PUFA	Sum n-3	Sum n-6	n-6/n-3
W. angusta 8878	35.4 (0.6)	3.86 (0.8)	60.8 (0.2)	40.2 (0.2)	20.6 (0.5)	0.51
W. arrhiza 8618	33.1 (0)	3.02 (0.7)	63.9 (0.3)	37.9 (0.3)	26.0 (0.4)	0.69
W. arrhiza 8853	35.4 (0.6)	3.26 (0.6)	61.4 (0.3)	32.6 (0.6)	28.8 (0.3)	0.89
W. arrhiza 9528	29.4 (0.7)	2.77 (8.7)	67.8 (0.3)	43.0 (0.2)	24.8 (0.4)	0.58
W. australiana 7540	34.9 (0.3)	2.79 (0.4)	62.3 (0.2)	42.1 (0.2)	20.2 (0)	0.48
W. borealis 9123	28.9 (1.4)	3.70 (0.3)	66.4 (0.6)	40.2 (0.5)	26.2 (0.8)	0.65
W. brasiliensis 7925	37.5 (1.3)	2.07 (7.0)	60.4 (1.0)	36.6 (1.1)	23.8 (0.8)	0.65
W. columbiana 7155	31.4 (1.3)	2.21 (1.4)	66.4 (0.5)	42.5 (0.7)	23.9 (0.4)	0.56
W. cylindracea 9056	34.9 (0)	3.46 (1.2)	61.6 (0)	36.7 (0)	25.0 (0)	0.68
W. elongata 9188	32.9 (0.6)	7.37 (1.2)	59.8 (0.2)	30.8 (0.3)	29.0 (0.3)	0.94
W. globosa 5514	34.7 (0.3)	4.09 (3.4)	61.2 (0.3)	37.0 (0.3)	24.2 (0.4)	0.66
W. globosa 5515	32.7 (0.9)	3.88 (0.5)	63.4 (0.5)	37.4 (0.3)	26.0 (0.8)	0.69
W. globosa 5537	32.6 (0.6)	4.11 (2.7)	63.3 (0.3)	36.8 (0.3)	26.5 (0.4)	0.72
W. globosa 9498	42.0 (0.9)	3.49 (0)	54.5 (0.7)	28.1 (0.7)	26.4 (0.8)	0.94
W. microscopica 2005	33.3 (3.6)	1.79 (1.1)	65.0 (1.8)	37.1 (1.9)	27.9 (2.5)	0.75
W. neglecta 9149	33.0 (1.5)	3.65 (0.8)	63.3 (0.9)	35.4 (1.1)	27.9 (0.7)	0.79
Average	33.9	3.47	62.6	37.2	25.4	0.70
± SD	3.1	1.3	3.2	4.1	2.5	0.14

Data were given as average of the sums of specific fatty acids (cf. ***Tables 3****,* ***4*** *for the single fatty acid content). As matrix-specific accuracy (RSD) does not exist, relative standard deviations (%) of the single sum of fatty acids were given in brackets. For averages ± SD cf.* ***Table 2****. SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.*

mg/100 g FDW), followed by (*all-E*)-β-carotene (ca. 10–30 mg/100 g FDW). The other carotenoids were present in much lower concentrations. The α-tocopherol content was between 0.5 and 13 mg/100 g FDW. A large variety of the contents of carotenoids and α-tocopherol in the different *Wolffia* species was observed as demonstrated by high standard deviations of

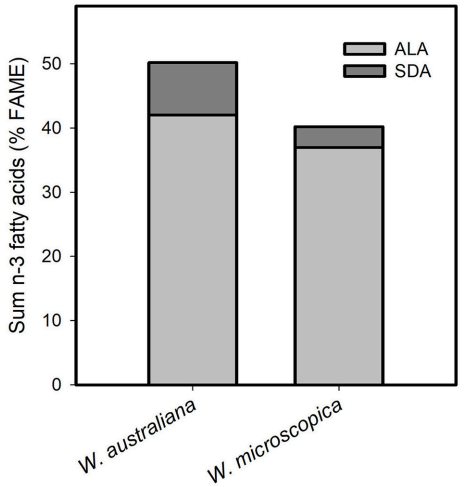

FIGURE 3 | Content of the fatty acids α-linolenic acid (ALA) and stearidonic acid (SDA) in the two species *W. australiana* 7540 and *W. microscopica* 2005. Data were given as sum of the two *n*-3 fatty acids in relation to fatty acid methyl ester (FAME, %).

TABLE 6 | Macro elements and ash content [g/kg FDW] in species of *Wolffia*.

Clone	Ca	*K*	Mg	Na	*P*	Ash content
W. angusta 8878	14.0	46.7	2.2	0.25	12.1	127
W. arrhiza 8618	25.7	88.1	2.39	0.18	18.8	229
W. arrhiza 8853	23.1	62.9	3.06	0.38	16.5	174
W. arrhiza 9528	19.8	93.7	2.66	0.34	16.7	224
W. australiana 7540	14.1	87.6	2.8	0.37	12.0	209
W. borealis 9123	18.1	66.3	2.59	0.17	13.4	164
W. brasiliensis 7925	13.9	84.1	2.32	0.12	14.5	190
W. columbiana 7155	26.0	82.4	2.74	0.66	20.9	216
W. cylindracea 9056	22.1	93.8	3.46	0.26	17.2	222
W. elongata 9188	32.5	61.5	4.55	0.22	21.3	198
W. globosa 5514	25.2	52.7	3.17	0.12	16.7	160
W. globosa 5515	24.1	54.6	2.99	0.11	17.1	159
W. globosa 5537	21.3	52.3	2.77	0.11	16.9	147
W. globosa 9498	13.4	42.1	1.96	0.13	13.5	105
W. microscopica 2005	20.6	76.8	4.07	0.38	18.9	206
W. neglecta 9149	12.2	39.7	1.91	0.13	12.5	166
RSD (%)	3.2	3.6	3.0	4.1	2.0	0.44
Average	20.4	67.8	2.85	0.25	16.2	181
± SD	5.8	18.8	0.71	0.15	3.0	37

For further explanations see ***Table 2****.*

TABLE 7 | Content of microelements in species of *Wolffia* [mg/kg FDW].

Clone	Fe	Mn	Cu	Zn	I	Se
W. angusta 8878	0.26	215	3.69	70.6	0.45	< 0.030
W. arrhiza 8618	0.16	78.4	3.31	50.0	0.31	< 0.030
W. arrhiza 8853	0.29	147	2.70	22.5	0.20	< 0.030
W. arrhiza 9528	0.20	144	2.89	40.7	0.25	< 0.030
W. australiana 7540	0.16	275	1.49	26.2	0.28	< 0.030
W. borealis 9123	0.19	249	2.70	33.4	0.44	< 0.030
W. brasiliensis 7925	0.13	330	2.96	31.4	0.20	< 0.030
W. columbiana 7155	0.13	80.4	3.43	69.6	0.92	< 0.030
W. cylindracea 9056	0.28	431	2.88	55.8	0.25	< 0.030
W. elongata 9188	0.31	336	2.34	65.8	0.51	< 0.030
W. globosa 5514	0.26	296	2.99	38.0	0.36	< 0.030
W. globosa 5515	0.12	302	4.43	41.2	0.20	< 0.030
W. globosa 5537	0.11	255	2.32	47.3	0.50	< 0.030
W. globosa 9498	0.37	203	2.50	84.2	0.50	< 0.030
W. microscopica 2005	0.33	136	3.83	79.4	0.56	< 0.030
W. neglecta 9149	0.40	200	2.39	92.4	0.27	< 0.030
RSD (%)	4.0	3.3	3.8	3.9	5.2	12.2
Average	0.23	230	2.93	53.0	0.39	< 0.030
± SD	0.09	98	0.70	21.7	0.19	–

For further explanations see ***Table 2.***

TABLE 8 | Heavy metals including As in species of *Wolffia*.

Clone	Cd	Pb	Hg	As
W. angusta 8878	81	1020	13	31
W. arrhiza 8618	13	100	17	85
W. arrhiza 8853	8.7	57	13	26
W. arrhiza 9528	11	52	13	60
W. australiana 7540	9	13	21	69
W. borealis 9123	51	120	15	64
W. brasiliensis 7925	14	23	22	39
W. columbiana 7155	590	410	18	69
W. cylindracea 9056	18	28	13	76
W. elongata 9188	63	89	27	52
W. globosa 5514	18	79	14	74
W. globosa 5515	15	71	17	35
W. globosa 5537	120	93	21	42
W. globosa 9498	15	610	17	42
W. microscopica 2005	180	460	24	42
W. neglecta 9149	17	680	18	45
RSD (%)	5.6	7.5	8.0	6.3
Average	76	244	18	53
± SD	145	301	4	18

For definition of the lead-group of heavy metals, cf. Appenroth (2010). Data are given in μg/kg FDW. For further explanations see ***Table 2****.*

the averages (**Table 9**). Impressive were the different contents of carotenoids and α-tocopherol in the different *Wolffia* species. For example, α-tocopherol content in *W. arrhiza* 8618 was ca. 13 mg/100 g FDW, while most other plants had contents of α-tocopherol lower than 5 mg/100 g FDW.

Phytosterols, Phytol, And Dihydrophytol

Total sterol content was between 2.4 and 5.3% of the total fat content in the *Wolffia* samples (**Table 10**). The main component of sterols was clearly β-sitosterol ranging from 57 to 84% of the total sterols. The contents of campesterol (mean: 8.5% of total sterols) and stigmasterol (mean: 7.7% of total sterols) were much lower but the differences between the different plant samples were very large as indicated by large standard deviations of the

TABLE 9 | Carotenoid and α-tocopherol contents in species of *Wolffia* [mg/100 g FDW] n.d., not detectable (<0.2 mg/100 g FDW).

Clone	(*all-E*)-β-Carotene	(*9Z*)-β-Carotene	(*13Z*)-β-Carotene	(all-E)-Lutein	(*all-E*)-Zeaxanthin	α-Tocopherol
W. angusta 8878	16.7	3.5	0.85	59.5	2.1	2.9
W. arrhiza 8618	23.8	5.7	6.50	70.2	2.1	12.8
W. arrhiza 8853	27.3	5.8	1.43	62.0	2.9	3.3
W. arrhiza 9528	17.2	3.8	0.84	51.8	2.1	7.3
W. australiana 7540	29.7	6.5	1.76	79.4	2.1	3.2
W. borealis 9123	17.8	3.9	0.98	53.3	1.54	0.82
W. brasiliensis 7921	19.9	4.4	1.00	47.5	2.1	2.3
W. columbiana 7155	24.8	5.4	1.28	75.2	3.5	2.7
W. cylindracea 9056	12.3	2.7	n.d.	51.2	1.91	5.6
W. elongata 9188	33.0	6.9	1.98	78.5	1.76	4.6
W. globosa 5514	19.1	4.1	1.00	58.1	1.85	4.1
W. globosa 5515	11.4	2.4	0.67	41.3	1.82	3.2
W. globosa 5537	17.6	3.7	1.03	46.4	1.89	7.9
W. globosa 9498	11.0	2.4	n.d.	43.3	1.59	3.5
W. microscopica 2005	20.6	4.5	1.10	66.7	1.64	0.51
W. neglecta 9149	16.1	3.4	1.01	59.3	1.41	3.5
RSD (%)	10.0	10.0	10.0	10.0	10.0	20.0
Average	19.9	4.3	1.5	59.0	2.0	4.3
± SD	6.4	1.4	1.5	12.2	0.5	3.0

The limit of detection (LOD) was calculated as three times the noise signal of a blank. For further explanations see ***Table 2****.*

averages. Stigmasterol could not be detected in *W. arrhiza* 8618 but had a share of 20% of the total sterols in *W. australiana* 7540. It is worth mentioning that also the other phytosterols, although in most cases present only in low amounts, showed very high variations between the different *Wolffia* species. Variation among the duckweed samples concerning the phytosterol content could not only be observed between the different species but also between the different clones of *W. arrhiza* (e.g., stigmasterol) and *W. globosa* (e.g., campesterol), although all plants were cultivated under identical conditions. During analysis of the phytosterol fraction we also noted two abundant peaks in the early part of the GC/MS chromatogram which were identified as 3,7*R*,11*R*,15-tetramethylhexadec-2E-enol (phytol) and the related dihydrophytol (Schröder et al., 2014). The isoprenoid alcohol phytol is usually bound to chlorophyll, which was also the source in the present samples. Concentrations of phytol (1.8–5.2% of the total fat content in the *Wolffia* samples) were in the range of the phytosterols, while the content of dihydrophytol was around 1% or lower (**Table 10**). Phytol has been detected in various plant oils but never at concentrations higher than 50 mg/100 g oil (Schröder et al., 2014).

DISCUSSION

In our recently published study (Appenroth et al., 2017), we investigated the protein, fat and starch contents, amino acid and fatty acid distribution of six duckweed species encompassing all five genera (Sree et al., 2016) in order to get an overview of the nutritional properties of duckweeds. We selected *W. microscopica* 2005 to investigate also the content of minerals, carotenoids and α-tocopherol as well as phytosterols and fiber. Knowledge of the composition of these plants is essential for potential users for human nutrition and to fulfill the judicial requirements of the novel food regulation during applications in the future. In order to deepen our understanding of the nutritional value of these tiny plant species, we selected 16 clones, all belonging to the genus *Wolffia,* comprising all the eleven species known till-date and investigated the above-mentioned components in the duckweed samples. This makes it possible to search for those *Wolffia* species or even clones of the same species that may represent valuable food sources for human nutrition.

In line with our previous investigation (Appenroth et al., 2017) reporting about the total protein content of the six species belonging to the five genera, the total protein content of the *Wolffia* species analyzed in the present study was in the similar range. *W. microscopica* 2005 was investigated in both projects and it contained slightly less protein and slightly more starch in the present study than in the recently published (Appenroth et al., 2017). This might be due to the longer cultivation period and the modification of growth medium. The fat content was low in general, but in contrast to FDW, starch, and fiber, the fat content varied between the different species. Even clones of the same species, such as *W. arrhiza* and *W. globosa*, showed significant differences in the total fat content. However, more important than the differences in the quantity of the different biochemical components, is the nutritional quality of these components. In our previous publication (Appenroth et al., 2017), we compared already the quality and quantity of nutritional components in several duckweeds with other plant species (cf. also Edelman and Colt, 2016) and demonstrated the advantages of duckweed especially concerning protein and fat quality.

TABLE 10 | Sterols and phytols in *Wolffia* species.

Clone	Total sterols	Phytol	Dihydro-phytol	Campesterol	Stigmasterol	β-Sitosterol	Sitostanol	Δ5-Avena-sterol	Δ7-Sitosterol	24-Methylene cycloartanol
W. angusta 8878	2.8	3.4	0.2	11	12	75	n.d.	2	n.d.	n.d.
W. arrhiza 8618	5.2	1.8	n.d.	9	n.d.	84	7	n.d.	n.d.	n.d.
W. arrhiza 8853	4.3	3.9	0.7	8	5	73	14[a]	n.q.	n.d.	n.d.
W. arrhiza 9528	4.6	4.8	0.5	11	2	77	10[a]	n.q.	n.d.	n.d.
W. australiana 7540*	3.8	4.9	0.5	7	20	61	n.d.	6	n.d.	2
W. borealis 9123	3.4	4.5	1.3	8	4	81	n.d.	3	n.d.	4
W. brasiliensis 7925	3.7	5.2	n.d.	7	17	73	n.d.	3	n.d.	n.d.
W. columbiana 7155*	4.4	4.7	n.d.	5	1	73	n.d.	n.q.[b]	17	n.d.
W. cylindracea 9056*	4.6	3.6	0.4	8	1	77	11[a]	n.q.	n.d.	2
W. elongata 9188*	5.3	3.8	0.7	13	17	57	n.d.	n.q.[b]	n.d.	7
W. globosa 5514	3.8	4.8	0.5	7	4	79	n.d.	3	2	5
W. globosa 5515	3.2	2.5	0.6	6	5	78	n.d.	2	3	6
W. globosa 5537	3.8	3.3	1.1	5	3	82	n.d.	3	2	5
W. globosa 9498	2.4	2.3	1.1	12	5	77	n.d.	6	n.d.	n.d.
W. microscopica 2005*	3.6	3.9	0.5	12	15	60	n.d.	9	n.d.	3
W. neglecta 9149*	3.9	3.0	0.5	7	4	80	n.d.	4	n.d.	n.d.
RSD (%)	7	6	4	4	4	1	3	5	3	3
Average	4.1	3.9	0.9	8	7	70	9	4	5	4
± SD	1.1	1.1	0.9	3	6	19	4	2	6	2

Total sterols, phytol and dihydrophytol are given in g/100 g fat. LOD = 0.007 g/100 g fat, LOQ = 0.02 g/100 g fat. Sterol components were given as % of total sterols. n.d., not detectable; n.q., not quantifiable. Limit of detection (LOD) and Limit of quantification (LOQ) were calculated as three and ten times the noise signal of a blank. For further explanations see ***Table 2****.*
*[a]Coelution with Δ5-avenasterol, [b]Coelution with β-amyrin (∼4–5 mg/g fat) *further minor sterols contributing up to 6% to the total sterol content which are not listed here were 24-methylene cholesterol, cycloartenol, Δ7-stigmastenol and Δ5,24(25)-stigmastadienol*
Method precision <10%, calculated for each substance as relative standard deviation (RSD) within the actual measurement concentration ($n_{sample} = 2$; $2 \leq n_{batch} \leq 16$)

$$RSD_{batch}[\%] = \overline{RSD_{sample}} = \frac{1}{n_{batch}} \sum_{i=1}^{n_{batch}} \frac{SD_{sample}}{\bar{x}} \cdot 100$$

Protein Content And Amino Acid Composition

In the set of 16 *Wolffia* samples that were investigated, *W. microscopica* played a special role by having a high protein quality with respect to human nutrition (cf. Appenroth et al., 2017). In comparison to the suggested reference values for essential amino acid requirements of preschool-age children [WHO (World Health Organization), 1985, 2007], all species and clones of *Wolffia* studied here showed excellent protein qualities. All ratios between the measured AA content and the reference (**Figure 2** and **Table S2**) were above 1 or close to 1 and *W. microscopica* is a top-scoring species. Interestingly, there are also differences between the different clones of *W. globosa* and *W. arrhiza*. Thus, we can also recommend the selection of more suitable clones for human nutrition.

Amado et al. (1980) published the most comprehensive screening for total protein content and amino acid composition of 26 species in a preliminary report. Beside a large number of clones of all other genera, the authors studied 24 clones of seven *Wolffia* species out of the nine species known at that time (Landolt, 1980; Sree et al., 2016). The authors reported analytical difficulties for the determination of sulfur-containing AA, which might explain why they called their study preliminary. Taken together, the findings of Amado et al. (1980) and Appenroth et al. (2017), and the study presented here cover the genus *Spirodela* with 11 clones, *Landoltia* with five clones, *Lemna* with 39 clones, *Wolffiella* with 20 clones and *Wolffia* with 40 clones. Together, it can be concluded that a high protein content and the quality of the amino acid spectrum of duckweed makes many of these plants suitable for human nutrition.

Fat Content And Fatty Acid Distribution

We have recently shown that the quality of the fatty acid profile of *Wolffia* covers nicely the requirements of human nutrition, although the fat content is generally rather low (Appenroth et al., 2017). In the present study, we found that the contribution of PUFA was 60% or higher for all analyzed *Wolffia* species, except for *W. globosa* 9498 (∼54.5% PUFA). Tang et al. (2015) investigated the fatty acid contents of the four species, *Spirodela polyrhiza*, *Landoltia punctata*, *Lemna aequinoctialis,* and *W. globosa* isolated from the lake Chao, China. Yan et al. (2013) reported the most comprehensive survey of fatty acids in duckweeds by investigating 30 species (one clone per species) including eight species of *Wolffia*. Unfortunately, the plants were cultivated mixotrophically (i.e., in the presence of sugar), which increases evidently the total fat content but makes it practically useless in a biotechnological context (Appenroth et al., 2017).

The main PUFA in the *Wolffia* species was the n-3 PUFA ALA with contents ranging from 28 to 43% FAME (**Table 4**). The content of the n-3 PUFA SDA is also worth mentioning

(**Figure 3**). This fatty acid is of high importance for human nutrition; it is already Δ-6 desaturated, has four double bonds and can be improved, i.e., metabolized, to the long-chain n-3 PUFA eicosapentaenoic acid (C20:5-c5,8,11,14,17) and docosapentaenoic acid (C22:5-c,4,7,10,13,16) in humans (Kuhnt et al., 2014, 2016; Dittrich et al., 2015). Within the genus *Wolffia* we detected SDA only in *W. microscopica* 2005 and *W. australiana* 7540. This makes these two species interesting for human nutrition as well as fundamental for nutrition research. In humans, the long-chain n-3 PUFA are also metabolized from ALA, which is the major fatty acid in the lipid fraction of the studied *Wolffia* species, whereby the conversion from SDA is more effective (Dittrich et al., 2015). The long-chain n-3 PUFA acts as anti-inflammatory and can therefore support the therapy of chronic inflammatory diseases such as rheumatoid arthritis (Dawczynski et al., 2017).

The major n-6 PUFA detected in the *Wolffia* species were LA and GLA, both fatty acids together represent nearly 30% FAME (**Table 4**). Beside the PUFA content, the n-6/n-3 ratio is important for human nutrition, because there is an imbalance marked by high intake of n-6 PUFA from plant oils such sunflower and soya oil, grains, sausage, and meat. On the other hand, the intake of n-3 PUFA do not reach the recommendations. This problem is of particular importance for the increasing number of vegetarians and vegans, which ban fish and meat.

Due to the enormous ALA content, the n-6/n-3 ratio was below 1.0 in all *Wolffia* species studied. According to the recommendations of the Food and Agriculture Organization of the United Nations (FAO, 2010), the n-6/n-3 ratio should not be >5 in human nutrition. Simoupolos (2006) reported that in Western diets the n-6/n-3 ratio is usually at least 15:1 to 17:1. A high n-6/n-3 ratio promotes the pathogenesis of many diseases, including cardiovascular diseases, cancer, and osteoporosis as well as inflammatory and autoimmune diseases. Thus, the generally high proportions of n-3 fatty acids of duckweed (Appenroth et al., 2017) and detected in all *Wolffia* species analyzed in this study could lower this ratio and therefore may contribute to a healthier human diet. *W. globosa*, which has a lower n-6/n-3 ratio than most other *Wolffia* species, is already used for human nutrition in several Asian countries.

Starch And Fiber Content

The starch content of the *Wolffia* species investigated in this study was 10 to 15% and is higher than in our previous study (Appenroth et al., 2017). This is, at least in part, caused by the slightly different cultivation conditions (i.e., especially longer cultivation time), as even the clone *W. microscopica* 2005 had higher starch content in the present study than measured before. However, the four clones of *W. globosa* showed different starch content although the cultivation conditions were identical. Therefore, the genetic constitutions must also influence starch production and accumulation. In summary, the starch content was low in all plant samples investigated, including the clones of *W. globosa*, which is already used for human nutrition. This low content of starch fits the requirements in Western countries to reduce high carbohydrate intake. In several developing countries, the low starch content has no disadvantages as human food, as starch intake is often high in these countries because of the staple food rice and maize [EFSA (European Food Safety Authority), 2017].

The fiber content in the investigated samples was similar and amounted to 26.2 ± 1.2% of FDW (**Figure 1**). This high content of dietary fiber is beneficial with respect to improving the Western diet by low-energy food components. Moreover, a high intake of dietary fiber is associated with a control of cardiovascular risk factors, such as total cholesterol and LDL cholesterol, and will therefore contribute to prevention and therapy of cardiovascular diseases, which are the major cause of death in the European countries (Kelly et al., 2017).

Mineral Composition

The mineral composition of all *Wolffia* samples investigated can be characterized as relatively rich in potassium and iron, and poor in sodium, making the duckweeds useful for healthy human nutrition. Duckweed samples contained the trace elements manganese, zinc and copper, although copper was not supplied through the nutrient medium. It might have accumulated from the impurities of supplemented macronutrients. The contents of minerals can be varied within a wide range by the application of suitable concentrations of the mineral content in the nutrient medium. This effect has not yet been investigated systematically in the context of optimizing intake of mineral nutrients for humans, but it can be expected that plants with high contents of selenium, zinc, or iodine can be easily produced with optimized nutrient media. Interestingly, different clones of the same species had different mineral contents although cultivated under identical conditions. For example, the iron content ranged between 0.11 and 0.37 g/kg FDW in the different clones of *W. arrhiza* and between 0.10 and 0.29 g/kg FDW in *W. globosa*. This means that also a genetic influence exists, resulting in different contents of trace elements and minerals under identical cultivation conditions.

Carotenoids and Vitamin E

The main carotenoids (*all-E*)-lutein and (*all-E*)-β-carotene, showed remarkably high contents and variations among the different *Wolffia* species. Together with the high values of zeaxanthin, this finding is important for the prevention of age-related macular degeneration, especially in comparison with other vegetarian food sources (Chew et al., 2013; Westphal and Böhm, 2015). *Wolffia australiana* 7540 and *W. elongata* 9188 showed the highest contents of both lutein and β-carotene. The content of α-tocopherol varied in the different species. The highest value was measured in *W. arrhiza* 8618, whereas the two other clones of this species had either medium (*W. arrhiza* 9528) or even low (*W. arrhiza* 8853) contents of α-tocopherol, again indicating high intraspecific genetic variation. Further, the role of light intensity or photoperiod remains to be investigated in this context in *Wolffia* species.

Phytosterols

Phytosterols have several effects on human health (Kritchevsky and Chen, 2005; Jahreis et al., 2013). Our results show that

Wolffia species are a rich source of phytosterols, such as β-sitosterol, campesterol, and stigmasterol, which contribute to the valuable nutrient profile of duckweeds. Moreover, it is evident that there are large differences between the different *Wolffia* species. This holds true especially for the minor components 24-methylenecholesterol, Δ5-avenasterol, Δ5,24(25)-stigmadienol, Δ7-sitosterol, Δ7-stigmasterol, cycloartenol and 24-methylenecycloartanol. *W. arrhiza* 8618 had the highest content of β-sitosterol, *W. australiana* 7540 had the highest content of stigmasterol, *W. elongata* 9188 had the highest content of campesterol and *W. arrhiza* 8853 had the highest levels of sitostanol and Δ5-avenasterol. In comparison with other *Wolffia* species, there seems to be no specific advantage of *W. globosa*, which is widely used for human nutrition in some Asian countries. The three investigated clones of *W. arrhiza* and the four clones of *W. globosa* illustrate that the content of phytosterols also depends on the origin of the clones and not only on the species.

Yield of Nutritional Components

For the intended practical application as human food, growth rates of the *Wolffia* plants as given in **Table 1** are important (see also Sree et al., 2015). As the yields of dry weight indicate, the differences were large and ranged from 5.4 g per g per week to almost 130 g per g per week. The high content of dietary fiber is beneficial in improving the Western diet by adding low-energy food components. Consequently, the plants with the highest growth rate have also the highest yield in protein, fat and starch (**Supplementary Table S3**). The dominating plant is *W. microscopica* 2005, as already shown in our previous publication (Appenroth et al., 2017). This species represents the fastest growing angiosperm of all (Sree et al., 2015; Sree et al., unpublished). There were also large differences in the yield between clones of the same species, i.e., *W. arrhiza* and *W. globosa* (Sree et al., 2015). In summary, our data show that it is worth to extend the investigations on *Wolffia* species including different clones to evaluate their potential for practical applications as human food.

AUTHOR CONTRIBUTIONS

K-JA, KSS, and GJ drafted the project. GJ coordinated analyses and evaluation of the data. K-JA and KSS produced the plant material. K-JA and GJ wrote the first draft of the manuscript. KSS analyzed the starch content. MB identified the plant species and measured growth rates. JE, SL, CD, CS, and GL analyzed and evaluated the fatty acid content. VB analyzed tocopherol and carotenoids. KS and WV analyzed phytosterols and phytols. KT-B and ML analyzed amino acids. RK and ML minerals. All authors read and revised the manuscript and agreed with the text.

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SUPPLEMENTARY MATERIAL

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Mass Production of *Lemna minor* and Its Amino Acid and Fatty Acid Profiles

Rina Chakrabarti[1], William D. Clark[2], Jai Gopal Sharma[3], Ravi Kumar Goswami[1], Avanish Kumar Shrivastav[3] and Douglas R. Tocher[2]*

[1] Aqua Research Lab, Department of Zoology, University of Delhi, New Delhi, India, [2] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, Scotland, [3] Department of Biotechnology, Delhi Technological University, New Delhi, India

The surface floating duckweed *Lemna minor* (Lemnaceae) is a potential ingredient to replace the application of fish-meal in the aqua-feed. The culture technique of the duckweed was standardized in outdoor tanks and then applied in the pond. Three consecutive experiments were conducted in tanks (1.2 × 0.35 × 0.3 m). In experiment 1, four different manures were used. In manure 1 (organic manure, OM) and manure 3 (2x OM), cattle manure, poultry droppings, and mustard oil cake (1:1:1) were used; in manure 2 (inorganic fertilizer, IF), urea, potash, triple superphosphate were used; manure 4 (2x OM+IF) was a combination of manure 2 and manure 3. In experiment 2, manure 1 (OM) and manure 2 (IF) were used, and manure 3 (OM+IF) was a combination of both manures. In experiment 3, OM and IF were selected. In pond (20 × 10 × 0.5 m), OM was applied. Fresh duckweed was seeded after 5 days of manure application. In experiments 1 and 3, total production was significantly ($P < 0.05$) higher in OM compared to other treatments. In experiment 2, there was no significant ($P > 0.05$) difference in production between OM and IF. In pond, relative growth rate (RGR) of duckweed ranged from 0.422 to 0.073 g/g/day and total production was 702.5 Kg/ha/month (dry weight). Protein, lipid, and ash contents were higher in duckweed cultured in OM compared to IF. The duckweed was a rich source of essential (39.20%), non-essential (53.64%), and non-proteinogenic (7.13%) amino acids. Among essential amino acids, leucine, isoleucine, and valine constituted 48.67%. Glutamic acid was 25.87% of total non-essential amino acids. Citrulline, hydroxiproline, taurine, etc. were found in the duckweed. The fatty acid composition was dominated by PUFA, 60–63% of total fatty acids, largely α-linolenic acid (LNA, 18:3n-3) at around 41 to 47% and linoleic acid (LA, 18:2n-6) at 17–18%. The nutritional value of duckweeds and their production potential in the pond conditions were evaluated. Duckweed biomass may thus be used to replace commercial fish-meal that is currently used in aquaculture.

Keywords: ***Lemna minor*, organic manure, proximate composition, amino acids, fatty acids**

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****Correspondence:***
Rina Chakrabarti
aquaresearchlab@yahoo.co.in

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INTRODUCTION

The surface floating macrophyte duckweed *Lemna* is the largest genus of the family Lemnaceae. They are abundant in the tropical and subtropical countries; growing profusely in still, nutrient-rich small ponds, ditches, and swamps or in slowly moving water bodies. The entire plant body consists of metabolically active non-structural tissue (Wolverton and McDonald, 1980) and the low fiber content of the plant has a beneficial impact on digestibility when used in animal feed. Duckweed grows on water with relatively high levels of N, P, and K and concentrates the minerals and synthesizes protein. The reported presence of various essential (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, tyrosine) and non-essential amino acids (FAO, 2001), poly-unsaturated fatty acids (Yan et al., 2013), β-carotene, and xanthophylls has made *Lemna* spp. a potential feed source for livestock (Skillicorn et al., 1993; Leng et al., 1995). In Taiwan, duckweeds are used as food for pig and poultry (FAO, 2001). In fish feed, *Lemna* spp. are usually used in fresh state. In recent years, there is a growing interest in this free floating macrophyte in the aqua-feed industry (for production of pelleted diets) to replace the protein-rich and costly fish-meal. Chakrabarti (2017) reported the production potential of duckweeds in freshwater bodies. Duckweeds are also used for treatment of waste water (Culley and Epps, 1973; Sutton and Ornes, 1975, 1977) and production of bio-fuel (Jarvis et al., 1988; Zhao et al., 2012, 2014, 2015a,b).

Protein plays a significant role in fish nutrition. Fish-meal is one of the commonly used protein-rich ingredients in the aqua-feed industry. Non-availability of quality fish meal and competition for the same resources with the terrestrial live-stock industry has made aquaculturists search for economically viable alternative protein sources. The alternative protein source (to fish meal) should be available in the required amount, cost-effective and preferably non-conventional to avoid competition with other uses and industries. The amino acid profile of the ingredient should meet the nutritional requirement of the cultivable species and prepared feed should be palatable and digestible to the fish. Digestibility test of duckweeds in carps and tilapia showed promising results (Hassan and Chakrabarti, 2009). Sharma et al. (2016) reported that the protein content of *Lemna minor* was 39.75 ± 0.47% and that digestibility of this plant protein for rohu *Labeo rohita* and common carp *Cyprinus carpio* was high as determined by an *in vitro* digestibility study.

The application of *Lemna* spp. as potential aqua-feed ingredients requires continuous production. Sustainable production of this plant requires an understanding of its nutritional and environmental requirements. The nutritional value of a plant also depends on the culture medium. The growth rate of duckweed clones in different natural (Rejmankova, 1975) and laboratory (Landolt, 1957) conditions varied. Many studies showed the production of *Lemna* spp. in domestic waste water (Zirschky and Reed, 1988), septage-fed ponds (Edwards et al., 1990, 1992), and effluent water (Vroon and Weller, 1995). The production of *Lemna* spp. in clean water with a known manuring schedule is required for commercial aqua-feed production. Few studies have been conducted to find the best balance of nutrients that may provide maximum growth of duckweed (FAO, 1989), especially for *Lemna* spp. The requirement to fertilize duckweeds depends on the source of the water. Rainwater collected in ponds may need a balanced NPK application. In Bangladesh, inorganic fertilizers (IFs, urea, triple superphosphate, and potash) were used for the production of duckweeds (DWRP, 1998). Hassan and Chakrabarti (2009) suggested a wide range of organic waste materials *viz.* animal manure, kitchen wastes, wastes from a wide range of food processing plants, biogas effluents, etc. for the production of duckweeds. A periodic supply of nutrients helped to avoid nutrient deficiency in the culture systems (Sutton and Ornes, 1975; Said et al., 1979). A direct relationship was found between the crude protein content of duckweed and the nitrogen content of the culture system. Although many species survive at extreme temperature, warm and sunny conditions are preferable for faster growth of the plants (Skillicorn et al., 1993). The distribution of various members of duckweed has been influenced by the microclimatic factors such as light intensity, salinity, and regional temperature (Landolt, 1986). The growth of duckweed is largely a function of environmental temperature and light, nutrient status of the culture medium and the degree of crowding of the plants (Hassan and Chakrabarti, 2009).

The present investigation aimed to develop a suitable culture technique for the production of *L. minor* in a sustainable manner. In our earlier study, it was found that the application of organic manures (OM) viz. cattle manure, poultry wastes, and mustard oil-cake was very effective in the mass production of live food organisms (Srivastava et al., 2006). Application of these manures helped in the large scale production of zooplankton in the outdoor facility (Chakrabarti and Sharma, 2008). These manures are easily available. Therefore, in the present study, these OMs were selected along with the other IFs to evaluate their effect on the production of duckweed. The culture technique was first standardized in a small outdoor facility under controlled conditions using various organic and IFs. The best method was then adopted in pond conditions to evaluate the large scale production potential of the macrophyte. The nutritional value of the produced plant was determined to evaluate its suitability as a potential feed ingredient for the aqua-feed industry.

RESULTS

Culture of *L. minor* in Outdoor Tanks

Water Quality

Three consecutive experiments were conducted in outdoor tanks to generate the baseline data for the production of duckweed *L. minor* in the pond conditions. Four, three, and two different manures were used in experiments 1, 2, and 3, respectively. In the pond experiment, only OM were applied for the production of *L. minor*. In experiment 1, water temperature ranged from 28.0 ± 0.4 to 25.5 ± 0.3°C in various treatments during September–October 2016. The intensity of light was recorded as 7,353 ± 138 to 4,642 ± 114 lux during this period in different culture tanks. There was no significant ($P > 0.05$) difference in water temperature (**Figure 1A**) and light intensity (**Figure 2A**) among various culture tanks. Water temperature and light intensity were higher at the beginning of the study and

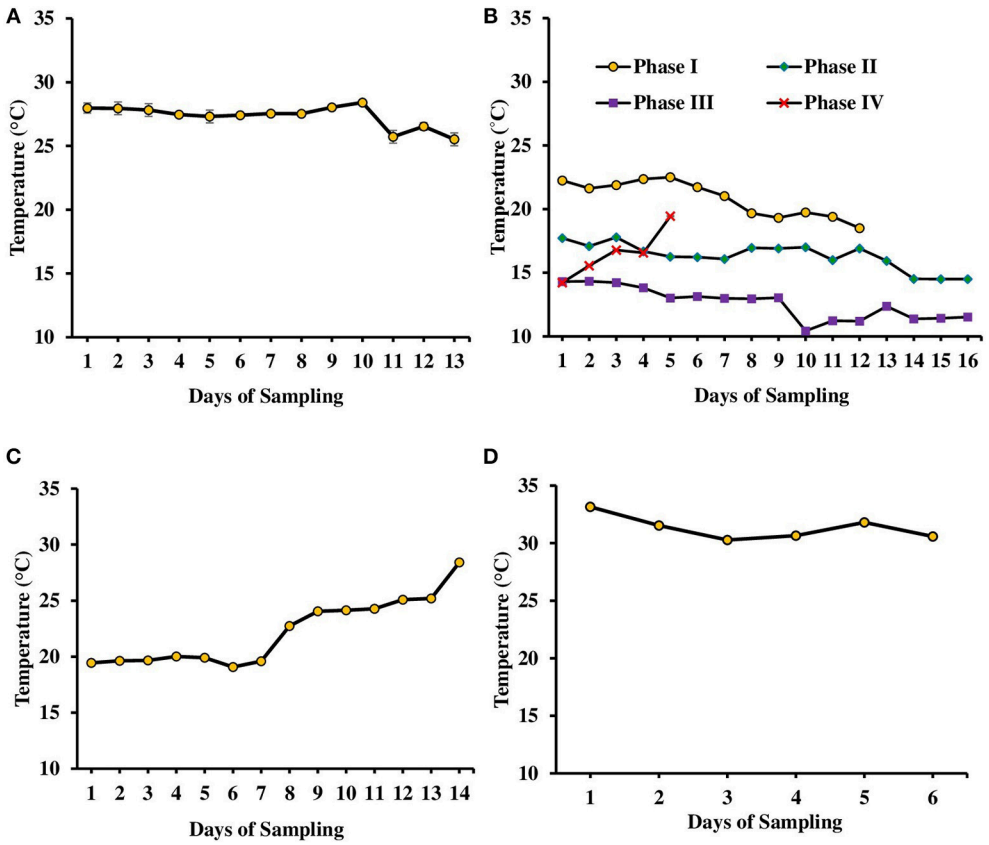

FIGURE 1 | Variation in water temperature found in tanks (experiments 1–3) and ponds during various days of culture of *L. minor*. **(A)** Experiment 1. **(B)** Experiment 2 has been divided in four phases depending on temperature—Phase I (October–November 2016), Phase II (November–December 2016), Phase III (December 2016–January 2017), and Phase IV (January–February 2017). The water temperature ranged from 22.5 to 18.5, 17.7 to 14.5, 14.3 to 11.2, and 14.2 to 19.4°C in first, second, third, and fourth phases, respectively. **(C)** Experiment 3 and **(D)** in ponds. There was no significant difference in temperature among various treatments of each experiment. Therefore, data for individual day of all treatments are presented as Mean ± SE. Data were collected at 9.00 a.m.

gradually reduced. The pH of water ranged from 7.10 to 7.41, 7.30 to 7.80, 6.96 to 7.35, and 6.99 to 7.52 in manures 1, 2, 3, and 4, respectively, throughout the study period. Dissolved oxygen level was significantly ($P < 0.05$) higher in the culture system fertilized with IFs compared to the other treatments throughout the study period. Dissolved oxygen levels in other treatments were always less than one (**Figure 3A**). Ammonia (NH_3) level was significantly ($P < 0.05$) higher in 2x OM+IF treatment compared to the others throughout the study period (**Figure 4A**). Among these four treatments, lowest ammonia level was found in IF. Ammonia level ranged from 0.585 to 4.65, 0.03 to 0.51, 8.4 to 22.6, and 15.65 to 42.97 mg/l in OM, IF, 2x OM, and 2x OM+IF, respectively, throughout the study period. Nitrite level was significantly ($P < 0.05$) higher in IF compared to the other treatments (**Figure 5A**). Highest level was recorded on day-1 of study in this treatment. Nitrite levels were 0.008 ± 0.002, 0.084 ± 0.024, 0.025 ± 0.008, and 0.033 ± 0.009 mg/l in OM, IF, 2x OM, and 2x OM+IF, respectively. Nitrate level was significantly ($P < 0.05$) higher in IF compared to the other treatments (**Figure 6A**). Nitrate levels were 1.30 ± 0.52, 15.30 ± 0.80, 5.23 ± 1.20, and 5.87 ± 1.22 mg/l in OM, IF, 2x OM, and 2x OM+IF, respectively. This showed the rate of nitrification among various treatments. Phosphate level was significantly ($P < 0.05$) higher in 2x OM+IF compared to the other treatments. This group was followed by 2x OM, OM, and minimum level was found in IF (**Figure 7A**). Highest level of phosphate was recorded on day-1 of study compared to the other days regardless of treatments. Conductivity was significantly ($P < 0.05$) higher in 2x OM+IF compared to the other treatments (**Figure 8A**). This group was followed by 2x OM, OM, and minimum level was found in IF.

The second experiment was conducted during October 2016–February 2017. In this experiment, seasonal effect on water quality and *L. minor* production was recorded (**Figure 1B**). Depending on the range of water temperature and light intensity the whole study period was divided into four phases. In the first phase (October–November) of the study, water temperature ranged from 22.5 to 18.5°C and then it reduced. Water temperature ranged from 17.7 to 14.5, 14.3 to 11.2, and 14.2 to 19.4°C in second (November–December), third (December–January), and fourth (January–February) phases, respectively. Light intensity also varied significantly in four different phases (**Figure 2B**). Light intensity ranged from 4,111 ± 232 to 2,322 ±

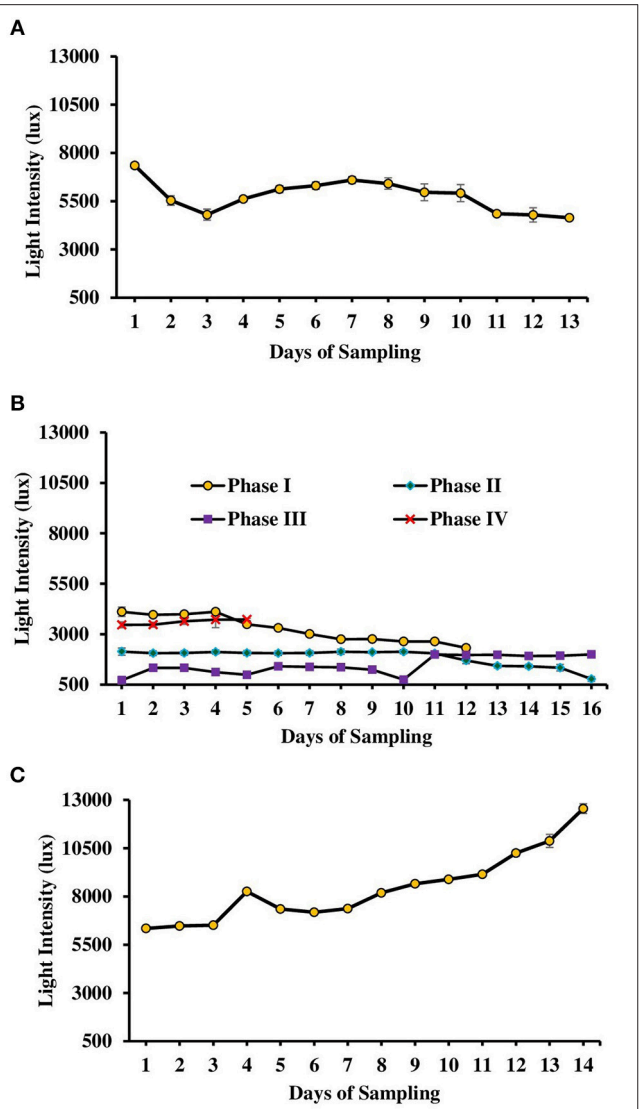

FIGURE 2 | Light intensity monitored during various days of culture of *L. minor* in tanks. **(A)** Experiment 1. **(B)** Experiment 2 has been divided in four phases depending on temperature—Phase I (October–November 2016), Phase II (November–December 2016), Phase III (December 2016–January 2017), and Phase IV (January–February 2017). **(C)** Experiment 3. There was no significant difference in light intensity among various treatments of each experiment. Therefore, data for individual day of all treatments were presented as Mean ± SE. Data were collected at 9.00 a.m. (Data for experiment 4 has not given.).

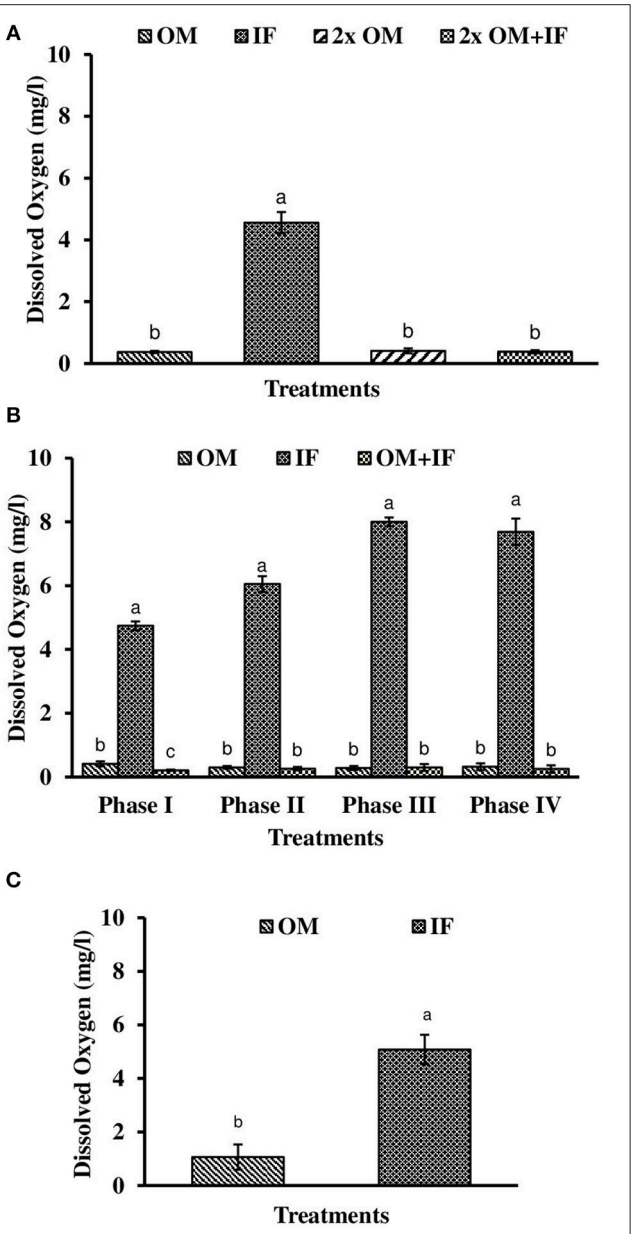

FIGURE 3 | Dissolved oxygen levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

130, 2,138 ± 178 to 781 ± 122, 718 ± 37 to 1,999 ± 34, and 3,463 ± 114 to 3,728 ± 57 lux in phase I, phase II, phase III, and phase IV, respectively. There was no significant ($P > 0.05$) difference in temperature and light intensity in various treatments throughout the study period. In OM, pH ranged from 7.19 to 7.60, 7.23 to 7.88, 7.52 to 7.91, and 7.66 to 8.13 in the first, second, third, and fourth phases, respectively. In IF, pH ranged from 7.50 to 7.86, 7.63 to 8.07, 7.68 to 8.05, and 7.60 to 8.09 in the first, second, third, and fourth phases, respectively. In OM+IF, pH ranged from 7.30 to 7.67, 7.32 to 7.86, 7.47 to 7.94, and 7.80 to 7.90 in the first, second, third, and fourth phases, respectively.

Dissolved oxygen level was significantly ($P < 0.05$) higher in IF compared to the other two treatments throughout the study period (**Figure 3B**). Dissolved oxygen level ranged from 4.7 to 8.7 mg/l in various days of study in IF. Dissolved oxygen level was <1 mg/l in most of the days of study in OM and OM+IF. Ammonia (NH_3) level was significantly ($P < 0.05$) higher in

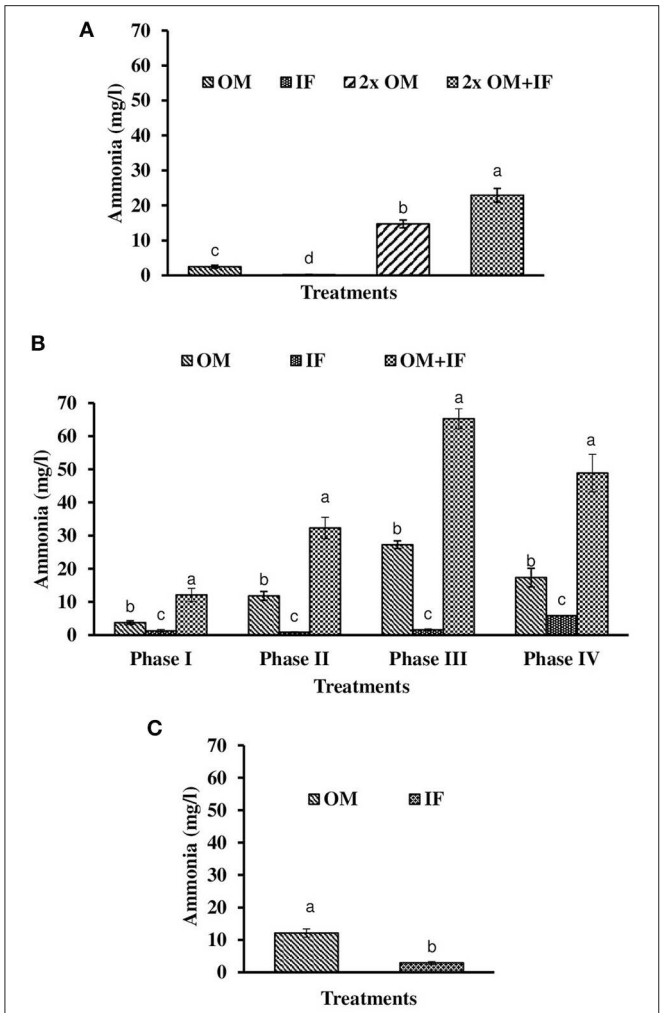

FIGURE 4 | Ammonia levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I−12, Phase II and III−16 each, and Phase IV−5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

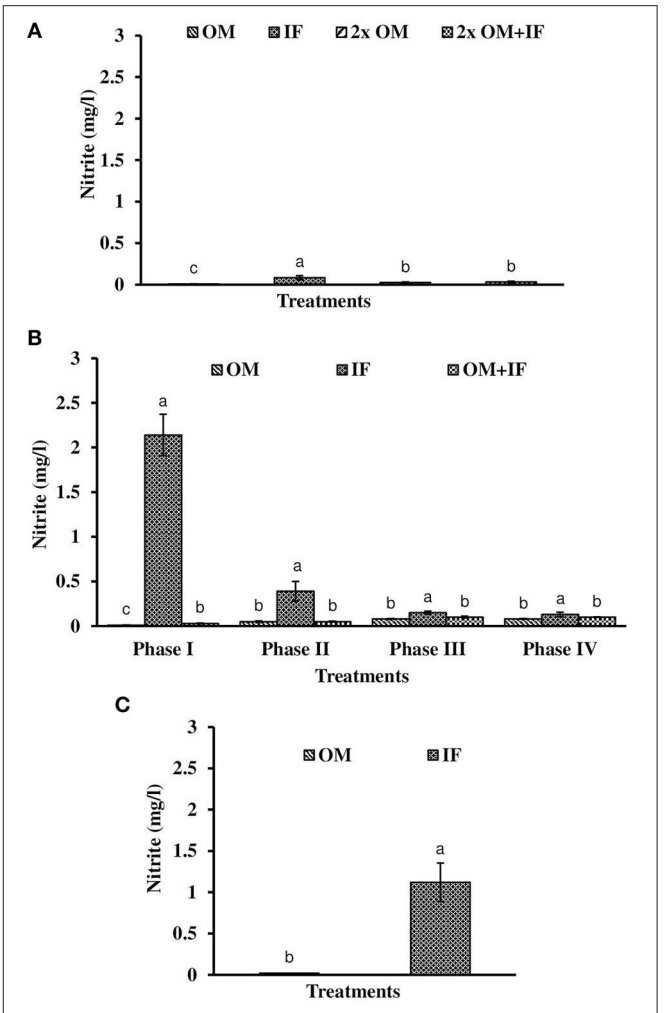

FIGURE 5 | Nitrite levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I−12, Phase II and III−16 each, and Phase IV−5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

OM+IF compared to the other two treatments in all four phases of the study (**Figure 4B**). In OM and OM+IF, highest ammonia level was found at phase III. In OM, ammonia level ranged 1.74 to 5.86, 4.61 to 21.06, 16.36 to 31.56, and 14.23 to 22.93 mg/l in the first, second, third, and fourth phases, respectively. In IF, ammonia level ranged from 0.20 to 2.88, 0.11 to 2.18, 0.57 to 5.37, and 5.80 to 5.92 mg/l in the first, second, third, and fourth phases, respectively. In OM+IF, ammonia level ranged from 5.42 to 19.96, 9.86 to 51.6, 47.70 to 88.5, and 42.96 to 60.16 mg/l in the first, second, third, and fourth phases, respectively. Nitrite level was significantly ($P < 0.05$) higher in IF compared to the other two treatments throughout the study period (**Figure 5B**). Nitrite levels were 0.01 to 0.08, 2.14 to 0.1, and 0.03 to 0.13 mg/l in OM, IF, and OM+IF, respectively, in all four phases. Nitrate level was significantly ($P < 0.05$) higher in IF compared to the other two treatments in first and second phases (**Figure 6B**). In OM+IF, significantly ($P < 0.05$) higher nitrate level was found in the third phase of the study. Nitrate levels were 0.16 to 1.95, 1.48 to 2.53, and 0.44 to 2.70 mg/l in OM, IF, and OM+IF, respectively, throughout the study period. Phosphate level was significantly ($P < 0.05$) lower in IF compared to the other two treatments throughout the study period (**Figure 7B**). In OM, phosphate level ranged 2.03 to 2.54, 1.22 to 2.22, 0.45 to 1.21, and 1.35 to 1.60 mg/l in the first, second, third, and fourth phases, respectively. In IF, phosphate level ranged from 0.15 to 0.42, 0.22 to 0.65, 0.30 to 0.55, and 0.28 to 0.45 mg/l in the first,

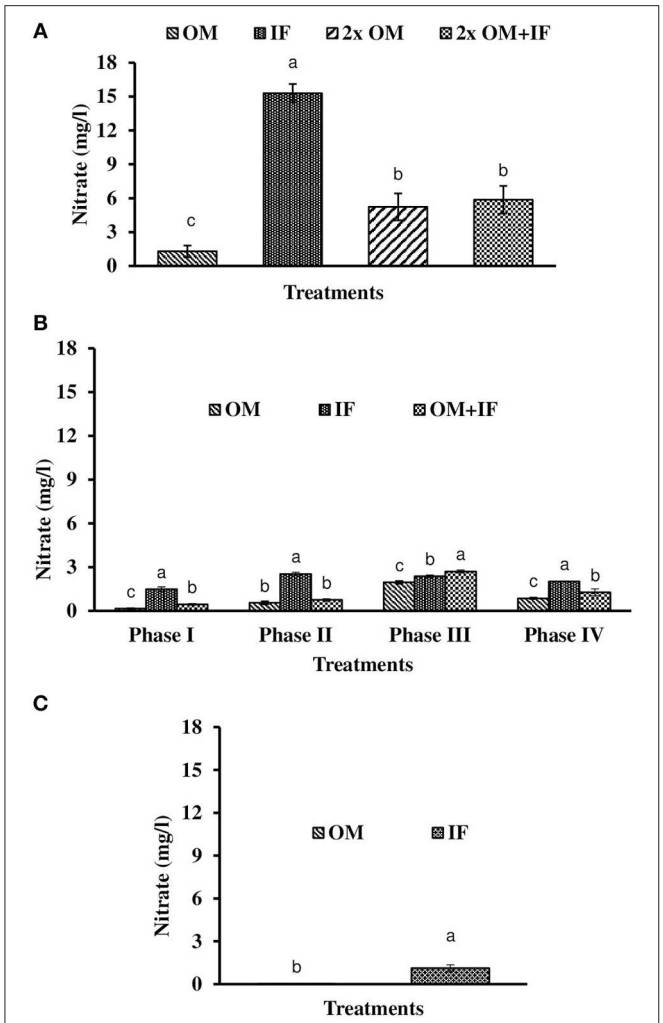

FIGURE 6 | Nitrate levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

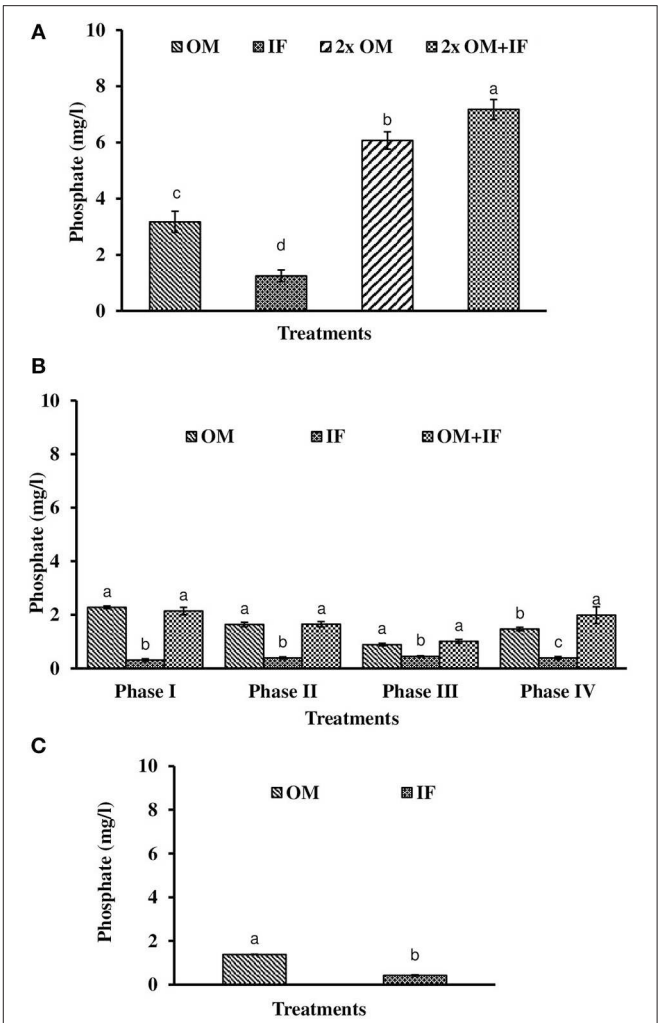

FIGURE 7 | Phosphate levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

second, third, and fourth phases, respectively. Conductivity was significantly ($P < 0.05$) higher in OM+IF compared to the other two treatments throughout the study period (**Figure 8B**). This group was followed by OM and IF.

The third experiment was conducted during February–April 2017. In this experiment, water temperature was minimum at the beginning and gradually increased ranging from 19.43 to 28.42°C. Light intensity also showed an increasing trend ranging from 6,341 ± 10 to 12,550 ± 283 lux throughout the study period. There was no significant ($P > 0.05$) difference in temperature (**Figure 1C**) and light intensity (**Figure 2C**) between the two treatments throughout the study period. The pH of the water ranged from 7.41 to 7.83 and 7.60 to 9.14 in OM and IF, respectively, during the study period. Dissolved oxygen level was significantly ($P < 0.05$) higher in IF compared to the OM throughout the study period. Dissolved oxygen level ranged from 1.27 to 0.12 mg/l in various days of study in OM. Dissolved oxygen level was always <1 mg/l in OM, except on the first day after manure application (**Figure 3C**).

Ammonia (NH_3) level was significantly ($P < 0.05$) higher in OM compared to IF throughout the study period (**Figure 4C**). Ammonia level ranged from 7.09 to 17.4 and 0.27 to 5.78 mg/l in OM and IF, respectively. Nitrite (**Figure 5C**) and nitrate (**Figure 5C**) levels were significantly ($P < 0.05$) higher in IF

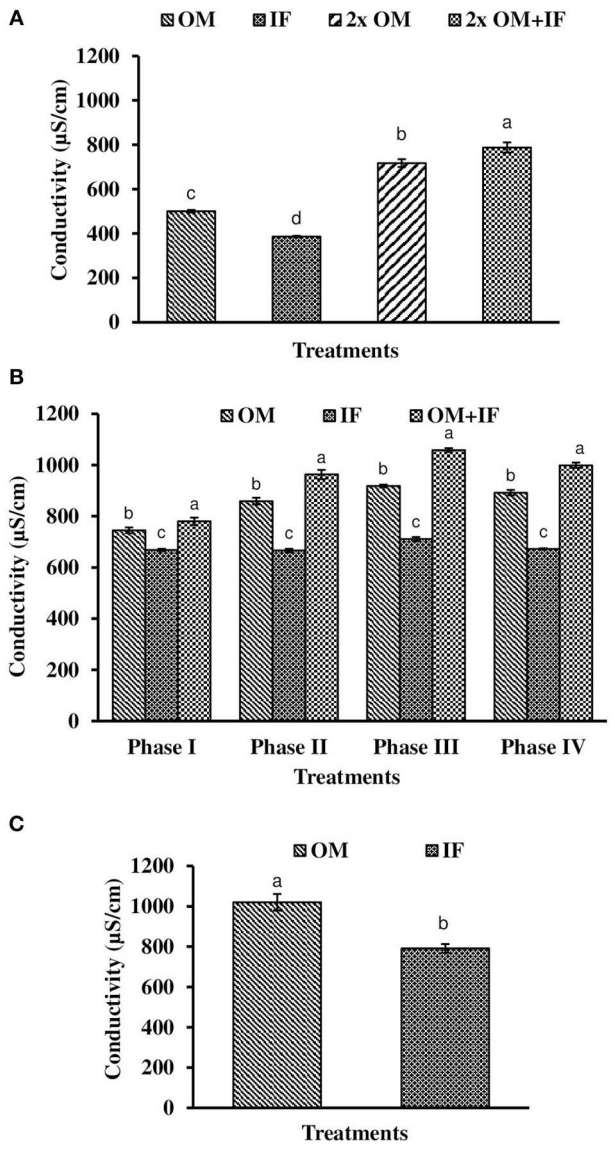

FIGURE 8 | Conductivity of water (in parenthesis) found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts are significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

compared to the OM throughout the study period. Nitrite level ranged from 0.008 to 0.04 and 0.11 to 2.66 mg/l in OM and IF, respectively, during the study period. Nitrate level ranged from 0.05 to 1.61 and 1.13 to 4.32 mg/l in OM and IF (**Figure 6C**), respectively. Phosphate level was significantly ($P < 0.05$) higher in OM compared to IF throughout the culture period. Phosphate level ranged 1.29 to 1.65 and 0.24 to 0.50 mg/l in OM and IF, respectively, throughout the study period (**Figure 7C**). Conductivity was significantly ($P < 0.05$) higher in OM compared to IF (**Figure 8C**).

Relative Growth Rate (RGR) and Production

The relative growth rate (RGR) of *L. minor* varied among different treatments in all three experiments. In experiment 1, *L. minor* was harvested 6, 5, 4, and 3 times in OM, IF, 2x OM, and 2x OM+IF, respectively, during 30 days of culture period. The RGR was always highest at first harvest regardless of treatments (**Figures 9A–C**). In experiment 1, RGR ranged from 0.521 to 0.047, 0.463 to 0.083, 0.239 to 0.034, and 0.215 to 0.078 g/g/day in OM, IF, 2x OM and 2x OM+IF, respectively, in various days of sampling. In experiment 2, plants were harvested 5, 8, and 3 times from OM, IF, and OM+IF, respectively. The RGR ranged from 0.23 to 0.014, 0.196 to 0.021, and 0.169 to 0.056 g/g/day in OM, IF, and OM+IF, respectively, in various days of sampling. In experiment 3, plants were harvested 7 and 3 times from OM and IF, respectively. The RGR ranged from 0.30 to 0.035 and 0.140 to 0.023 g/g/day in OM and IF in various days of sampling.

Production of *L. minor* in Outdoor Tanks

In all these study, macrophytes were harvested when the surface area of the tanks were filled with macrophytes. There was difference in harvesting time in various treatments due to differences in the growth of plants. In all these treatments, 50% of the total biomass was harvested at each harvest, except the final one. In experiment 1, production of macrophytes was significantly ($P < 0.05$) higher in OM compared to the other treatments (**Figure 10A**). Lowest production was recorded in 2x OM+IF throughout the study period.

In experiment 2, *L. minor* production was significantly ($P < 0.05$) higher in OM compared to the other two treatments in first and second phases. In third phase, macrophyte was harvested thrice from IF. There was no harvesting from OM as the growth of duckweed was very less and it was not covering the whole surface area of the tank. Plants were harvested only when they covered the whole water body. The growth of macrophytes was very poor in OM+IF in the third phase. There was no survival of plants in this treatment. In fourth phase, *L. minor* production was significantly ($P < 0.05$) higher in OM compared to IF. Total production was significantly ($P < 0.05$) higher in OM and IF compared to OM+IF treatment. There was no significant ($P > 0.05$) difference in total production between OM and IF (**Figure 10B**).

In experiment 3, *L. minor* production was significantly ($P < 0.05$) higher in OM compared to the IF (**Figure 10C**). In OM, macrophyte was first harvested after 14 days of inoculation of plants when the tank was totally covered with macrophytes. In IF, tanks were filled with macrophytes after 28 days of inoculation and then plants were harvested. The type of manures influenced the growth of macrophytes.

Culture of *L. minor* in Ponds

Water Quality

This experiment was conducted in cemented ponds during July–August 2017. The variations in water quality parameters reflected the seasonal variations as well as effect of manure application.

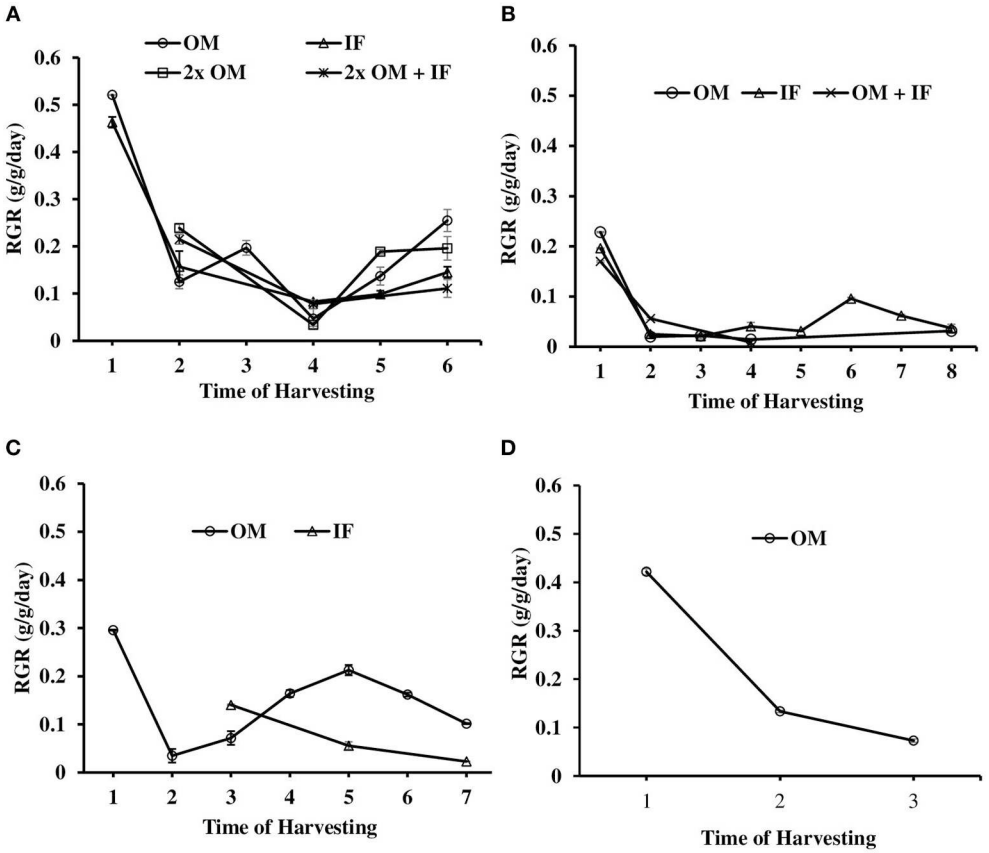

FIGURE 9 | Relative growth rate (RGR) of *L. minor* found in **(A)** experiment 1, **(B)** experiment 2, **(C)** experiment 3, and **(D)** ponds during various days of culture. In experiment 2, harvesting pattern was as follows: Phase I—first harvest, Phase II—second, third, and fourth harvests, Phase III—fifth, sixth, and seventh harvests, Phase IV—eighth harvest. In experiment 3, there was no harvesting in IF on 1, 2, 4, and 6 sampling. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

Water temperature and pH ranged from 33.15 to 30.27°C and 7.32 to 8.04, respectively, throughout the study period. Water temperature was higher in July and gradually it decreased (**Figure 1D**). Dissolved oxygen level ranged from 1.04 to 3.57 mg/l on various days of study. Dissolved oxygen level was higher at the beginning of the experiment; the level decreased after the application of manures and with the growth of macrophytes as it covered the surface area of the ponds. Ammonia, nitrite and nitrate levels ranged from 5.02 to 10.57, 0.003 to 0.12, and 0.23 to 2.44 mg/l, respectively. Phosphate level ranged 1.15 to 1.70 mg/l during the study period (**Table 1**). Conductivity ranged from 1,022 to 1,351 μS/cm throughout the culture period of duckweed. Ammonia, nitrite, nitrate, and phosphate levels varied with the days of manure application.

Relative Growth Rate (RGR) and Production

The RGRs of *L. minor* were 0.422, 0.133, and 0.073 g/g/day in first, second, and third harvest times, respectively (**Figure 9D**). The duckweed was first harvested after 10 days of introduction. Macrophyte was harvested thrice during 1 month culture period. Fifty percent macrophyte was harvested at the time of first and second harvesting and all plants were collected at the time of third harvest. The production of *L. minor* was 1.17 ± 0.005 Kg/m^2/month (**Figure 10D**). Total production of duckweed in the pond was 702.5 Kg/ha/month (dry weight).

Composition of *L. minor*

Proximate composition analysis of duckweed showed that there was difference between the macrophytes cultured with OM and IF. Protein, lipid, and ash contents were significantly ($P < 0.05$) higher in macrophytes cultured in OM compared to IF. Balancing these higher levels, carbohydrate content was lower in macrophytes cultured in OM compared to IF (**Table 2**).

The amino acid profile of duckweed cultured in OM showed some interesting results (**Table 3**). The essential (39.20%), non-essential (53.64%), and non-proteinogenic (7.13%) amino acids were present in duckweed. All essential amino acids viz. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine were found in adequate quantity. Leucine, isoleucine, and valine consisted 48.67% of the essential amino acids. Among non-essential amino acids, glutamic acid was 25.87%. Several non-proteinogenic amino acids viz. citrulline, hydroxiproline, taurine etc. were also present in the duckweed.

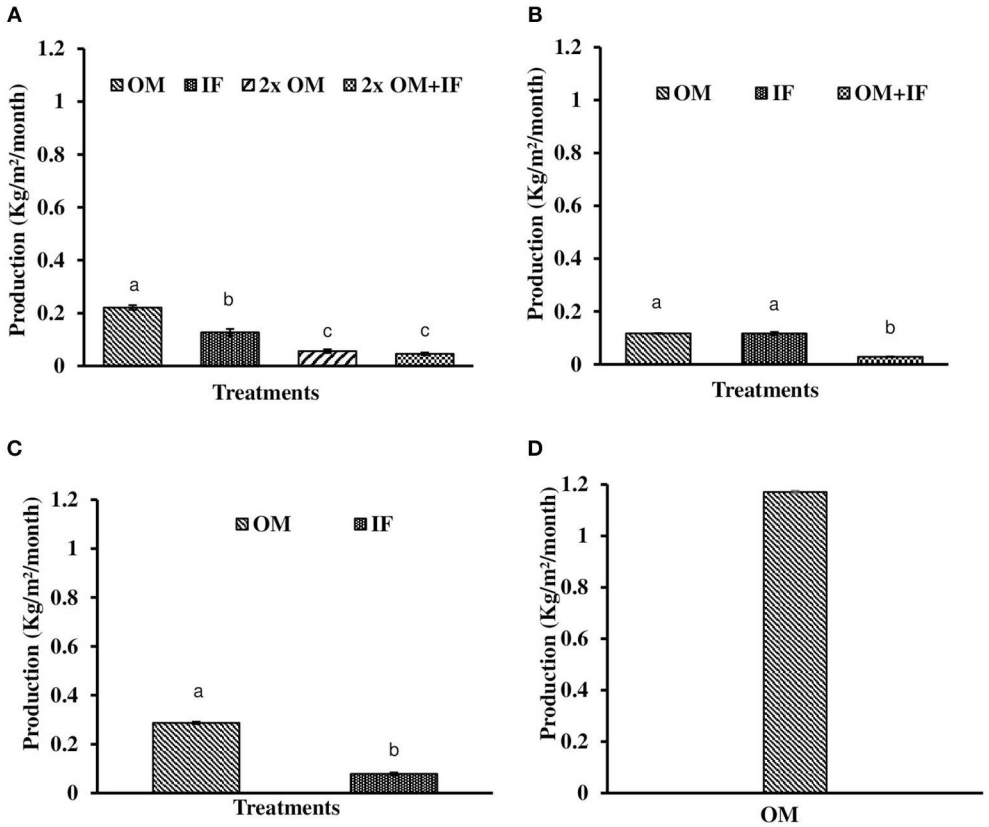

FIGURE 10 | Total production of *L. minor* found in **(A)** experiment 1, **(B)** experiment 2, **(C)** experiment 3, and **(D)** ponds. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 3$). OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

TABLE 1 | Culture conditions of *L. minor* in ponds.

Parameter	Range	Mean ± SE
Temperature (°C)	30.27–33.15	31.32 ± 1.0
pH	7.32–8.04	–
Dissolved oxygen (mg/l)	1.04–3.57	2.25 ± 0.52
Ammonia (mg/l)	5.02–10.57	3.25 ± 0.7
Nitrite (mg/l)	0.003–0.12	0.045 ± 0.01
Nitrate (mg/l)	0.23–2.44	1.13 ± 0.01
Phosphate (mg/l)	1.15–1.70	1.52 ± 0.07
Conductivity (μS/cm)	1,022–1,351	1,161 ± 1.7

The average values (three replicates) of 6 days of sampling were presented as Mean ± SE.

TABLE 2 | Proximate composition of *L. minor* (% of dry weight) grown in the tanks.

	Organic manure (OM)	Inorganic fertilizer (IF)
Protein	36.07 ± 0.18	27.12 ± 0.40*
Lipid	8.45 ± 0.61	7.15 ± 0.06
Ash	21.41 ± 0.20	19.42 ± 0.30*
Carbohydrate	34.07 ± 0.36	46.31 ± 0.74*

**Denotes significant difference ($P < 0.05$).*

The amino acid profile of duckweed cultured in IF was not provided.

The fatty acid composition of *L. minor* was dominated by PUFA, which accounted for 60–63% of total fatty acids, largely α-linolenic acid (LNA, 18:3n-3) at around 41–47% and linoleic acid (LA, 18:2n-6) at 17–18%, followed by saturated fatty acids (∼23–26%) and monoenes (11–12%) (**Table 4**). As with proximate composition, fatty acid profile was also influenced by manures, with *L. minor* grown in OM having significantly higher proportions of LA, LNA, saturated, and monounsaturated fatty acids, and total PUFA when compared to the inorganic counterpart (IF). Due to the higher lipid content of *L. minor* grown in OM, all fatty acids were found in higher absolute amounts (mg/100 g dry mass) in macrophytes grown in OMs. Irrespective of manure, the *L. minor* lipid profile contained no long-chain PUFA (LC-PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), although there was a trace of eicosapentaenoic acid (EPA, 20:5n-3).

DISCUSSION

In the present study, suitable manures and their dose for the production of *L. minor* was studied first in outdoor-tanks and

TABLE 3 | Amino acid profile of *L. minor* cultured with organic manures (OM).

Amino acids	Concentration (g/100 g)
ESSENTIAL	
Histidine (His)	0.894 ± 0.011
Isoleucine (Ile)	2.043 ± 0.064
Leucine (Lue)	4.132 ± 0.046
Lysine (Lys)	2.683 ± 0.161
Methionine (Met)	0.859 ± 0.014
Phenylalanine (Phe)	2.571 ± 0.034
Threonine (Thr)	1.924 ± 0.138
Tryptophan (Trp)	0.365 ± 0.010
Valine (Val)	2.664 ± 0.096
NON-ESSENTIAL	
Alanine (Ala)	2.882 ± 0.041
Arginine (Arg)	3.060 ± 0.045
Asparatate (Asp)	3.714 ± 0.372
Cysteine (Cys)	0.381 ± 0.032
Glutamic Acid (Glu)	6.427 ± 0.102
Glycine (Gly)	2.861 ± 0.031
Proline (Pro)	1.248 ± 0.035
Serine (Ser)	2.348 ± 0.333
Tyrosine (Tyr)	1.905 ± 0.125
NON-PROTEINOGENIC	
Phosphoserine (p-Ser)	0.578 ± 0.000
Taurine (Tau)	0.041 ± 0.015
Phospho ethanol amine (PEA)	0.023 ± 0.006
Sarcosine (Sar)	0.097 ± 0.004
α Amino adipic acid (a-AAA)	0.045 ± 0.013
α Amino-n-butaric acid (a-ABA)	0.150 ± 0.012
Cystathionine (Cysthi)	0.093 ± 0.019
β-Alanine (b-Ala)	0.111 ± 0.020
β-Amino isobutyric acid (b-AiBA)	0.971 ± 0.271
γ-Amino-n-butyric acid (g-ABA)	0.405 ± 0.014
Ethanol amine ($EOHNH_2$)	0.146 ± 0.004
Hydroxylysine (Hylys)	0.058 ± 0.007
Ornithine (Orn)	0.014 ± 0.001
1 Methylhistidine (1 Mehis)	0.087 ± 0.003
3 Methylhistidine (3 Mehis)	0.117 ± 0.004
Carnosine (Car)	0.106 ± 0.001
Hydroxy proline (Hypro)	0.133 ± 0.015
Citrulline (Cit)	0.126 ± 0.002

then the best condition was adopted in ponds. The results of three consecutive studies in outdoor systems showed that duckweed production was influenced by the quality of manures and doses, and environmental factors. Both organic and IFs were used separately and in combinations for the production of *L. minor*. In experiments 1 and 3, the average RGR of *L. minor* was higher in OM compared to IF. The average RGR-values of duckweeds in OM were same in experiment 1 in outdoor tanks and in the pond experiment (0.21 g/g/day). In experiment 1 and 3, total macrophytes production (**Figures 6A–C**) was significantly higher in OM compared to the other treatments; whereas in experiment 2, there was no significant difference in total production between OM and IF. The growth of duckweed was affected by low temperature in OM in the third phase (i.e., fifth, sixth, and seventh harvests) of experiment 2. Reduced growth of duckweed affected the total production. In contrast, in the culture system treated with IFs the growth was continued in the cold condition (**Figure 5B**). Production of macrophytes in 2x OM and 2x OM+IF of experiment 1, and OM+IF of experiment 2 were negligible. For this reason these manures were not adopted for the production of macrophytes and less discussed in the present study. Higher doses of manures resulted in significantly ($P < 0.05$) higher levels of ammonia that affected the growth and production of macrophytes in these treatments. RGR was always higher at first harvest regardless of treatments. In experiment 1, RGR in OM during second harvest was lower compared to 2x OM and 2x OM+IF as this was the first harvest for these two latter treatments. There was no production in all treatments, except OM at the time of third harvesting. Therefore, in experiment 1, the RGR was lower in OM during fourth harvest compared to the other treatments. Similarly, in experiment 3, the first harvest of *L. minor* from IF, was the third harvest for OM. The poor growth rate of duckweed resulted in slow production and delayed harvest. Availability of space and nutrient might influence the RGR in the first harvest compared to the successive harvests. The OMs are rich sources of nitrogen (N), phosphorous (P_2O_5), and potash (K_2O) and are usually applied in agricultural land in India (Gaur et al., 1990). The amount of nutrients of OM varied with season and geographical location. The mixture of these three manures fulfills the requirements of the plant. The decomposition of these manures enhances their availability to the plant. In the present study, mixture of manures was decomposed for 5 days before application in the water bodies.

Porath et al. (1979) reported the RGR of fresh *L. minor* cultured in both laboratory and field conditions. They obtained the highest value of 0.346 g/g/day in laboratory condition, whereas the value became 0.099 g/g/day in the field condition. The fresh yield of duckweed ranged from −0.026 to 0.66 Kg/m^2/week during 8 successive weeks of culture in the manured pond. Oron (1994) reported that the RGRs of duckweeds ranged from 0.10 to 0.35 g/g/day. RGR of *Lemma gibba* grown in desert ponds ranged from 0.081 to 0.191 g/g/day (Guy et al., 1990). Rejmankova (1975) observed 0.20 and 0.22 g/g/day RGR of *L. minor* and *L. gibba*, respectively, in field conditions. In the present study, RGR of duckweed was comparable with the earlier studies.

Hassan and Chakrabarti (2009) reported that variations in climatic conditions, nutritional status of the water body and differences in species resulted in the differences in the production of the macrophytes. Most of the data were generated from short-term studies in small-scale experimental systems. The data generated from longer duration study in commercial-sized systems are most wanted. In the present study, 702.5 Kg (dry mass)/ha/month (i.e., 8.43 tons/ha/year) *L. minor* were produced from pond with the application of OMs. In UASB effluent and nutrient non-limiting water, *L. minor* production were 10.7 and 16.1 tons (dry mass)/ha/year (Reddy and DeBusk, 1985; Vroon and Weller, 1995). In septage-fed pond, the production of *Lemna*

TABLE 4 | Fatty acid composition of *L. minor* as percentage of total fatty acids (%) or as mg fatty acids per 100 g dry weight (absolute).

	Organic manure (OM)		Inorganic fertilizer (IF)	
	Percentage	**Absolute**	**Percentage**	**Absolute**
14:0	1.10 ± 0.02	36.7 ± 3.3	1.14 ± 0.07	26.6 ± 1.0
15:0	0.31 ± 0.03	10.1 ± 0.4	0.36 ± 0.04	8.4 ± 0.22*
16:0	19.14 ± 0.03	634.8 ± 43.0	22.21 ± 4.32	516.7 ± 51.4
17:0	20.47 ± 0.11	79.5 ± 0.1	22.32 ± 2.49	77.7 ± 2.5
18:0	1.08 ± 0.01	35.7 ± 2.9	1.47 ± 0.57	33.9 ± 10.1
24:0	1.10 ± 0.00	36.4 ± 2.6	1.22 ± 0.17	28.4 ± 1.2
Total saturated	22.72 ± 0.03	753.7 ± 51.3	26.40 ± 5.17	613.9 ± 61.9
16:1n-9	5.60 ± 0.07	185.7 ± 10.6	5.36 ± 0.42	126.2 ± 21.8
16:1n-7	2.32 ± 0.02	77.0 ± 5.9	2.23 ± 0.12	52.4 ± 7.7
18:1n-9	2.15 ± 0.19	71.6 ± 11.3	2.88 ± 1.22	66.3 ± 22.3
18:1n-7	1.46 ± 0.03	48.4 ± 4.3	1.46 ± 0.04	34.3 ± 2.4
Total monoenes	11.53 ± 0.17	382.6 ± 32.2	11.93 ± 0.73	279.2 ± 9.7*
18:2n-6	16.88 ± 0.04	560.1 ± 40.1	18.07 ± 1.71	422.2 ± 0.6*
Total n-6 PUFA	16.88 ± 0.04	560.1 ± 40.1	18.07 ± 1.71	422.2 ± 0.6*
18:3n-3	46.35 ± 0.07	1,537.5 ± 108.9	41.24 ± 7.16	976.2 ± 260.9
20:5n-3	0.14 ± 0.02	4.5 ± 0.3	0.14 ± 0.02	3.6 ± 0.5
Total n-3 PUFA	46.49 ± 0.13	1,542.0 ± 102.6	41.38 ± 7.36	979.8 ± 265.9
Total DMA	2.37 ± 0.04	78.6 ± 4.1	2.23 ± 0.24	52.6 ± 10.7
Total PUFA	63.38 ± 0.10	2,102.1 ± 142.6	59.45 ± 5.66	1,402.0 ± 266.5
Total fatty acids		3,317.0 ± 230.3		2,347.6 ± 225.0

Results: mean ± SD. DMA, dimethyl acetals; PUFA, polyunsaturated fatty acids.
**Denotes significant difference between OM and IF ($P < 0.05$).*

perpusilla was 11.2 tons (dry mass)/ha/year (Edwards et al., 1990). It was suggested that in an aquatic environment with sufficient nutrients and optimum environmental conditions around 10–20 tons (dry mass)/ha/year duckweeds can be harvested (Hassan and Chakrabarti, 2009). Seeding of the plant also played important role in the production. DWRP (1998) recommended 40 Kg/100 m^2 for *L. minor* in order to obtain a dense cover in 3 days. But getting this amount of *L. minor* from control culture system for seeding was difficult. Therefore, only 1 Kg (wet weight) of plant was seeded in the 200 m^2 pond in the present study. The growth rate of *L. minor* was 3–6 folds higher at the time of first harvest compared to the second and third harvests. Optimum water quality parameters *viz*. temperature, ammonia, phosphate levels etc. influenced the growth of the macrophyte. In the month of July, the presence of moisture in the air also influenced the macrophytes production (compared to the dry season). Moreover, there was enough space at the initial phase; due to the growth of the plants, there was competition for space and nutrients at the later phase.

The impact of various water quality parameters on the production of *L. minor* was documented in the present study. Temperature is known to be the master abiotic factor. In an outdoor facility, *L. minor* were cultured under a wide range of temperature of 11.5–28.4°C during September 2016–April 2017. In the OM-based culture system, production was greatly reduced as the water temperature become <18.5°C from November onwards. An increasing trend in duckweed production was recorded from February onwards as temperature rose to 19.4°C. In pond the experiment, 31.5–30.3°C temperature was favorable for duckweed production. Temperature tolerance and optima are species-specific. Maximum growth for most of the species of *L. minor* was obtained between 17.5 and 30°C (Culley et al., 1981; Gaigher and Short, 1986). The growth rate declined at low temperature. Reduced growth rate was found in some duckweed at the temperature below 17°C (Culley et al., 1981). Most species seemed to die at 35°C water temperature. All these studies showed that 17–18°C was the critical temperature and 27–31°C was optimum temperature for the production of *L. minor*.

The intensity of light also played major role in the production of *L. minor*. In the experiment 2, as light intensity reduced, particularly in the second and third phases (718–2,138 lux), production reduced drastically; an increasing trend was recorded as light intensity increased (3,463–3,728 lux) in experiment 2. In the present study, maximum *L. minor* production was recorded at 7,353–10,878 lux light intensity. Mkandawire and Dudel (2007) suggested 4,200–6,700 lux light intensity at 14–16 h photoperiod for the optimum production of *L. gibba* and *L. minor*.

Duckweeds have wide range of pH tolerance. The biomass of duckweeds doubled in 2–4 days at pH 7–8 (Culley et al., 1981). Khondker et al. (1994) showed that pH 6.9–7.8 was suitable for *L. perpusilla* production. The optimum growth of *L. perpusilla* was found at pH 7.36 (Van der Does and Klink, 1991). In the present study, pH ranged from 7.32 to 8.04 in all outdoor and

pond culture systems in OM and IF. Application of higher dose of OMs resulted in pH <7.0 in 2x OM and 2x OM+IF in experiment 1. The pH of water decreased after the application of OMs in all experiments and then increased with the duration of study.

In the production of *L. minor*, a direct effect of dissolved oxygen was not recorded. In OM, dissolved oxygen level was generally <1.0 mg/l, whereas a higher concentration of dissolved oxygen was always recorded in IF. But the production was lower in IF compared to OM in most of the harvestings (except third phase of experiment 2). Application of OMs reduced the oxygen level in the culture system. Dissolved oxygen might influence the nitrification of ammonia as higher level of nitrite and nitrate levels were found in IF compared to OM.

Ammonia (NH_3) level was always lowest in IF regardless of experiment. Leng et al. (1995) suggested that ionized ammonia (NH_4^+) as preferable nitrogenous substrate for *L. minor* culture. The ammonium-ammonia balance shifted toward the un-ionized (NH_3) form at alkaline pH. This resulted in higher concentration of free ammonia in the culture system which affected the duckweed production. Leng et al. (1995) also suggested that the ammonia concentrations of cultured water should be 7–12 mg N/l for the maintenance of crude protein content of duckweed. In the present study, higher level of ammonia (18.8–32.0 mg/l) also affected the production of duckweed in culture systems fertilized with OMs, especially during winter. In experiment 2, significantly ($P < 0.05$) higher level of ammonia (combined with low temperature) affected the growth of macrophytes in OM+IF treatment. Porath and Pollock (1982) suggested that ammonia (NH_4^+) uptake is temperature-sensitive in duckweed. Nitrite and nitrate levels were lowest in OM. Higher levels of nitrite and nitrate in IF showed the better nitrification rate in this treatment compared to the OM. Duckweeds preferred ammonia as nitrogen source compared to nitrate and grew better in presence of the former nutrient (Lüönd, 1980).

Phosphorus is one of the limiting nutrients (after nitrogen) and is essential for rapid growth of the macrophyte. In OM, phosphate level was most of the time >1.0 mg/l, whereas phosphate level was <1.0 mg/l in IF. This showed the phosphate limitation of this treatment. In some species of duckweeds, decreased growth rate was obtained at *P*-values <0.017 mg/l (Lüönd, 1980). In *L. perpusilla*, a positive correlation was recorded between the concentrations of phosphate and silicate and the biomass (Khondker et al., 1994). Phosphorous (PO_4-P) should range between 4 and 8 mg/l for the optimum production of duckweeds (Hassan and Chakrabarti, 2009). The present study showed that the production of *L. minor* was dependent on nutrient availability in terms of nitrogen and phosphorous and environmental factors like temperature and light intensity of the culture system.

Many studies showed the proximate composition of several species of duckweeds from different geographical areas. Protein content ranged from 14.0 to 23.5, 25.3 to 29.3, 9.4 to 38.5, and 26.3 to 45.5% in *L. minor* (Majid et al., 1992; Zaher et al., 1995), *L. perpusilla* (Hassan and Edwards, 1992), *L. gibba* (Hillman and Culley, 1978; Culley et al., 1981), and *Lemma paucicostata* (Mbagwu and Adenji, 1988), respectively. In the present study, 36.07 ± 0.18 and 27.12 ± 0.4% protein contents were found in *L. minor* cultured in OMs and IFs. The nutritional status of the water body influenced the crude protein content of the duckweed; protein content ranged from 9 to 20% in nutrient-poor water or under sub-optimum nutrient conditions, whereas it ranged 24–41% in nutrient-rich water. The crude protein content of duckweed increased up to 40% at ammonia concentration of 7–12 mg N/l (Leng et al., 1995). The protein content of *L. minor* collected from a natural pond of northeast region of India was 28.0 ± 1.7 (Kalita et al., 2007). Appenroth et al. (2017) reported that in different species of duckweeds protein contents ranged from 20 to 35%.

Presence of high quality protein was reported in various studies (Porath et al., 1979; Rusoff et al., 1980). The essential amino acid profile of duckweed was better compared to the most of the plant proteins and more closely resembled to animal protein. Guha (1997) reported that the protein of duckweeds was rich in certain amino acids that were often low in plant proteins. The nutritional value of duckweeds is comparable with alfalfa in terms of two essential amino acids—lysine and arginine. These are required in animal feeds. High amount of leucine, threonine, valine, isoleucine, and phenylalanine and less amount of methionine and tyrosine are found in duckweeds. The amino acid content of the *L. paucicostata* was equivalent to that of blood, soybean, and cottonseed meals and considerably exceeded that of groundnut meal (Mbagwu and Adenji, 1988). Amino acids like, lysine (4.8%), methionine and cystine (2.7%), and phenylalanine and tyrosine (7.7%) were also present in the duckweeds (Appenroth et al., 2017). In the present study, the amino acid composition of *L. minor* confirmed its nutritional value as feed ingredient as it is a rich source for essential and non-essential amino acids. The presence of non-proteinogenic amino acids viz. taurine, citrulline, hydroxyproline, sarcosine etc. enhanced the nutritional value of duckweed.

Like protein, lipid, and ash contents of *L. minor* grown in OM were higher compared to the macrophytes grown in IF in the present study. Culley et al. (1981) reported 6.3% ether extracts in *L. gibba* from USA. In different species of duckweeds fat contents ranged from 4 to 7% (Appenroth et al., 2017). Lipid content of *L. minor* was higher (7.15–8.45%) in the present study, but slightly lower than the 10.6% reported by Yan et al. (2013). The duckweeds produced in nutrient poor water bodies showed lower lipid content (1.8–2.5%) compared to the plant grown (3–7% lipid) in water enriched with nutrient (Hassan and Chakrabarti, 2009). The lipid and ash contents of *L. minor* grown in natural pond were 5.0 ± 0.1 and 25.0 ± 1.6%, respectively (Kalita et al., 2007). Ash contents of various species of *L. minor* ranged from 11.1 to 17.6% (Hassan and Edwards, 1992; Zaher et al., 1995). The ash content of *L. minor* ranged from 19.42 to 21.41% in the present study. Duckweeds are known to accumulate large amounts of minerals in their tissues. Higher amount of ash and fiber and lower amount of protein were found in duckweed colonies with slow growth rate (Skillicorn et al., 1993). The ash content of duckweed was not influenced by the nutrient status of water (Leng et al., 1995). The fatty acid content of *L. minor* cultured in the present study was generally similar to that measured by Yan et al. (2013), who reported a composition of around 25% saturated fatty acids, 5% monoenes and 70% PUFA,

with a very similar level of LA (16%) but a higher proportion of LNA at ∼54% of total fatty acids. The trace level of EPA found in the present study most likely simply reflects the presence of a very small amount of freshwater microalgae, which can often contain some EPA but rarely DHA, in the *L. minor* harvest. Negesse et al. (2009) also reported the presence of short chain fatty acids (SCFA, 16.6%) in *L. minor* (C2 11%, C3 3.1%, C4 1.4%, and C5 0.4%) that could be useful for commercial utilization of duckweed as they can serve as preservatives, preventing bacterial growth. In recent study, Appenroth et al. (2017) reported that in different species of duckweeds, polyunsaturated fatty acids content ranged from 48 to 71%; the high level of n3 fatty acids resulted in a favorable n6/n3 ratio and enhanced the nutritional value of the duckweeds.

CONCLUSIONS

The present study showed that *L. minor* can be produced using cheap and easily available OMs and the produced macrophytes are rich source of protein, lipid, and minerals. Amino acid profile and fatty acid profile confirmed the suitability of the macrophytes in the production of aqua-feed.

Among water quality parameters temperature, light intensity, pH, ammonia, phosphate, and conductivity played major role. These factors should be maintained within reasonable limits for survival and growth of the macrophytes. The management strategies for duckweed culture should focus on the time of manure application and harvesting.

MATERIALS AND METHODS

Culture of *L. minor* in Outdoor Tanks

Lemna minor was collected from a pond located in the Department of Botany, University of Delhi and the plant was identified based on the morphological characteristics (oval shaped fronds, 2–5 fronds remained together, presence of three nerves in each frond and cylindrical root sheath with two lateral wings) with the help of scientist of Department of Botany. Since then the macrophyte was cultured in the Department of Zoology. Three consecutive experiments were conducted to generate the baseline data for the production of *L. minor*. Macrophytes were grown in cemented tanks (1.2 × 0.35 m) maintained in the outdoor facility of Department of Zoology, University of Delhi. The depth of water was 30 cm throughout the study period. Dechlorinated tap water, supplied by the Municipal Corporation of Delhi was used for all experiments. The first experiment was conducted during September–October 2016 and the duration of the experiment was 30 days. Four different manures were used. In manure 1 (OM) and manure 3 (OM 2x OM), cattle manure (local), poultry droppings (local), and mustard oil cake (Double Hiran Mustard Oil-cake, Malook Chand Food Pvt. Ltd., Aligarh, U.P., India) (1:1:1) were used at the rate of 1.052 and 2.104 Kg/m^3 (Srivastava et al., 2006); in manure 2 (IF), urea (IFFCO, Indian Farmers Fertilizer Cooperative Limited, New Delhi, India), potash (Narmada, Gujarat Narmada Valley Fertilizers & Chemicals, Gujarat, India), triple superphosphate (IPL, Indian Potash Limited, Chennai, India) were used at the rate of 15, 3, and 3 Kg/ha/day, respectively, based on the study of DWRP (1998); manure 4 (2x OM+IF) was a combination of manure 2 and manure 3. The amount of IFs was calculated for 10 days and applied in the culture tank. In all these experiments, OMs were applied at the rate of one fourth dose of initial dose at every 10 days interval. In IFs, similar dose (initial amount) of manures was applied at every 10 days interval. Manures for individual tank were mixed with tap water and allowed to decompose for 5 days before application. All manures, except cattle manure were applied in dry conditions. The moisture content of cattle manure was measured and the weight was adjusted.

The second experiment was conducted during October 2016–February 2017 and the duration of the experiment was 105 days. The three different manures used in this experiment were selected based on the results of the first experiment. The first two manures, manure 1 (OM) and manure 2 (IF) were similar to the earlier experiment, and manure 3 (OM+IF) was a combination of manure 1 and manure 2. Third experiment was conducted during February–April 2017. In this experiment, manure 1 (OM) and manure 2 (IF) were selected. This selection was based on the production potential of these manures compared to the others.

Fresh *L. minor* (15 g wet weight) was seeded after 5 days of manure application in each tank. Three replicates were used for each treatment. Harvesting of *L. minor* started when the plant covered the whole surface area of the tank; 50% of the total production was harvested during first and other consecutive harvests; all macrophytes were harvested at the end of the study. Production was expressed as Kg/m^2/month on wet weight basis.

Culture of *L. minor* in the Ponds

In CIFE, Rohtak Center (Indian Council of Agricultural Research), Haryana three cemented ponds (200 m^2, 20 m × 10 m) were prepared for the culture of *L. minor* in June 2017. The bottom was cleaned thoroughly and the pond was filled with ground water (50 cm). The OMs, like cattle manure, poultry dropping, and mustard oil cake (1:1:1) were applied at the rate of 1.052 Kg/m^3 (Srivastava et al., 2006). Organic manures applied for the production of duckweed were selected based on the results of outdoor cemented tanks. Three replicates were used for the study. The culture conditions of *L. minor* developed in the tank experiments were also applied in the pond. All manures were mixed properly and allowed to decompose for 5 days. Then fresh *L. minor* cultured in cemented tanks of Department of Zoology, University of Delhi was seeded (wet weight) at the rate of 1 Kg/pond. It covered a small area of the pond. The experiment was continued for 30 days. Like outdoor tank experiments, one fourth dose of initial dose of manure was applied at every 10 days interval. Harvesting started when the plant covered the whole surface area of the pond. Total pond area (200 m^2) was divided in four quadrates of 50 m^2 each. In first and second harvests, *L. minor* was collected from two quadrants for 50% harvesting (**Figures 11A,B**). In third harvest, all plants were collected and air dried (**Figure 11C**). Production was expressed as Kg/m^2/month on wet weight basis.

Water Quality

Various water quality parameters were monitored regularly in outdoor tanks and in ponds at 9.00 a.m. Water temperature and pH (PHC 10101), conductivity (CDC 40101), dissolved oxygen (LDO 10101), ammonia, NH_3 (ISENH318101), and nitrate (ISENO318101) were measured using HACH multimeter (HQ 40d, USA). Light intensity was measured with probe (PMA 2130) attached with a lux meter (SOLAR LIGHT, PMA 2100, USA) at the surface of the water. Phosphate (4500-P D. Stannous Chloride Method) and nitrite (4500-NO_2^- B. Colorimetric Method) were measured following the method of APHA (2012).

Relative Growth Rate

The RGR of *L. minor* was calculated using the formula: RGR = ln $(W_t/W_0)/t$.

Where, W_t and W_0 are the fresh weight of macrophytes at the time of harvest (t) and at the time of introduction of plant (zero reference time), respectively; t is the time interval in days.

Fresh *L. minor* was used for the study and RGR-value was expressed as g/g/day, i.e., production (g) of *L. minor* from 1 g of starter culture of *L. minor* per day.

Composition of *L. minor*

Biochemical (proximate) compositions of *L. minor* cultured in tanks (OM and IF) were determined by standard methods (AOAC, 2000). Moisture contents were recorded after drying at 110°C for 24 h, and the ash fraction obtained after incineration at 600°C for 16 h. The crude protein content was determined by measuring nitrogen content (N × 6.25) by Kjeldahl analysis (Tecator Kjeltec 1030 analyser, Foss, Warrington, UK). The crude lipid content was measured gravimetrically after extraction by Soxhlet (Tecator Soxtec 2050, Warrington, UK). Carbohydrate content was then determined by the subtraction method.

Amino acid composition was estimated using Automatic Amino Acid Analyzer L-8900 (Hitachi Co. Ltd., Tokyo, Japan). Dry and powdered plant sample was hydrolyzed with 6 N HCl at 110°C for 22 h. Hydrolyzed sample was dried in Nitrogen Evaporator (PCi Analytic Private Limited, Maharashtra, India). Then 0.02 N HCl was added in the sample and concentration of protein in the sample was 0.5 mg/ml. The sample was kept in the Auto sampler. Sample injection volume was 20 µl. Methionine, cysteine, and tryptophan are destroyed during hydrolysis with 6 N HCl; these amino acids are treated with specific reagents. Methionine and cysteine were oxidized with performic acid and then treated with 48% hydrobromic acid. Macrophyte sample was hydrolyzed with 4 N methanesulfonic acid and 3-(2-aminoethyl) indole for the estimation of tryptophan. Rest of the methods were same for all amino acids. The ninhydrin derivative of proline and hydroxyproline was monitored at 440 nm, and other amino acids were monitored at 570 nm. The contents of detected amino acids were quantified by comparing their peak areas with those of authentic standards provided with the equipment. Amino Acids Mixture Standard Solutions, Type B and Type AN-2 (Wako Pure Chemical Industries, Limited) were used. Standard solutions for glutamine and tryptophan (Sigma-Aldrich, USA) were prepared before analysis.

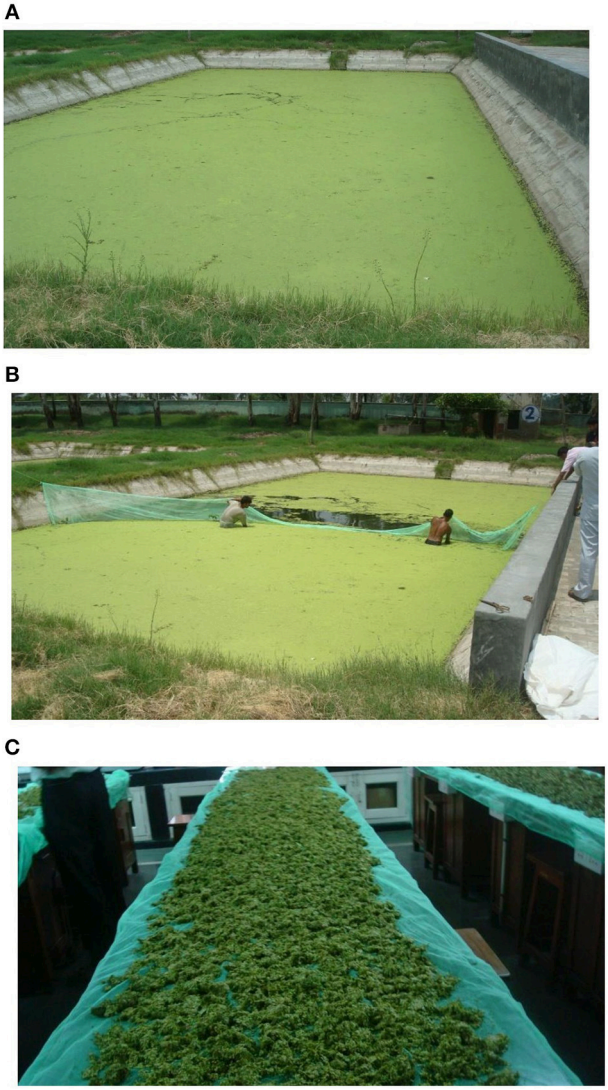

FIGURE 11 | (A) Production of *L. minor* after 10 days of culture in the pond of Central Institute of Fisheries Education, Rohtak, Haryana, India. **(B)** The harvesting (50%) of *L. minor* in pond. **(C)** Air drying of harvested duckweed at University of Delhi.

For fatty acid composition, *L. minor* samples were dried at 40°C and ground. The total lipid fraction was then extracted from 1 g of dried material by homogenization in chloroform/methanol (2:1, v/v) using a tissue disrupter (Ultra-Turrax, Fisher Scientific, Loughborough, UK), and lipid content determined by weighing (Folch et al., 1957). Fatty acid methyl esters (FAME) of total lipid were then prepared by acid-catalyzed transesterification for 16 h at 50°C (Christie, 2003). The FAME were extracted and purified as described in detail previously (Tocher and Harvie, 1988), and then separated and quantified by gas-liquid chromatography (Fisons GC-8160, Thermo Scientific, Milan, Italy) using a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), on-column injection

and ionization detection. Identification of FAMEs was by comparison to known standards and published data (Tocher and Harvie, 1988), and data collected and quantified using Chromcard for Windows (Thermoquest Italia S.p.A., Milan, Italy).

Statistical Analysis

Data were presented as mean ± SE unless otherwise stated. Data were analyzed using one-way analysis of variance (ANOVA), Duncan's multiple range test, DMR (Montgomery, 1984) and, where appropriate, by Student's *t*-test (SPSS software 19.0). Statistical significance was accepted at $P < 0.05$ level.

AUTHOR CONTRIBUTIONS

RC, DT, and JS designed the study; RG, AS, RC, and JS cultured the plant and analyzed samples; WC and DT analyzed samples; RC, DT, and JS wrote the manuscript; RG, WC, and AS prepared graphs and tables.

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Effect of Exogenous General Plant Growth Regulators on the Growth of the Duckweed *Lemna minor*

*Desi Utami†, Ami Kawahata†, Masayuki Sugawara†, Rahul N. Jog, Kyoko Miwa and Masaaki Morikawa**

Graduate School of Environmental Science, Hokkaido University, Sapporo, Japan

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***Correspondence:**
Masaaki Morikawa
morikawa@ees.hokudai.ac.jp

†Present Address:
Desi Utami, Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia
Ami Kawahata, R&D Center, HAYASHIBARA Co., Ltd., Okayama, Japan
Masayuki Sugawara, Graduate School of Life Sciences, Tohoku University, Sendai, Japan

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Gibberellic acid (GA_3), indole-3-acetic acid (IAA), salicylic acid (SA), abscidic acid (ABA), jasmonic acid (JA) 1-amino cyclopropane-1-carboxylic acid (ACC) and aminoethoxyvinylglycine (AVG) are popular growth regulators of plants. However, the effects of their exogenous addition on the biomass production of aquatic plants, including *Lemnoideae* plants, "duckweeds," are largely unknown. In this study, the growth of *Lemna minor* was tested for 10 d in Hoagland medium containing each compound at different concentrations of 0–50 μM. GA_3, IAA, and SA were found to have no apparent positive effect on the growth at all concentrations tested. Conversely, ACC and JA moderately and AVG and ABA severely inhibited the growth of *L. minor.* Among the tested compounds, ascorbic acid had an apparent growth-promoting effect.

Keywords: *Lemna minor,* plant growth regulators, gibberellic acid, indole-3-acetic acid, salicylic acid, 1-aminocyclopropane-1-carboxylic acid, aminoethoxyvinylglycine, ascorbic acid

INTRODUCTION

Duckweeds, a general name for plants belonging to the *Lemnoideae* subfamily, represent the smallest free-floating monocotyledonous aquatic plants with vascular systems. *Lemnoideae* includes five genera: *Landoltia*, *Lemna*, *Spirodela*, *Wolffiella,* and *Wolffia*. The plant body consists of "frond," leaf-like structure, and "root" (except for *Wolffiella* and *Wolffia*, which are rootless duckweeds). Some duckweeds develop seed-like "turion," which contains high starch and anthrocyanin depending on the temperature, light and nutrient conditions (Smart and Trewavas, 1983). Although duckweeds are flowering plants, they primarily reproduce asexually by vegetative propagation. Duckweeds are robust, environmentally well-adapted, and ubiquitously distributed from 55° S to 70° N. They were recently highlighted as a potential biomass.

The following are the advantages of duckweeds as biomass: (1) they grow at significantly high rates; (2) they grow on wastewater and uptake and remove contaminant minerals as nutrients; (3) they can be used as slow, but low-cost wastewater treatment systems driven by sun light; (4) they provide nonfood competitive biomass with high protein and starch, but low lignin and cellulose content; and (5) they change their constituent starch and protein content depending on the growth condition. Thus, duckweeds are expected to be one of the next-generation biomass resources to be associated with animal feed, biofuel and starch-based green chemistry (Cheng, 2010; Baliban et al., 2013). Increase in the growth rate of duckweeds should contribute to the increasing availability of duckweed biomass and allow the prevention of global warming and realization of sustainable industries and societies. Increasing attention is being paid to producing duckweeds coupled with wastewater treatment (Xu et al., 2012; Zhao et al., 2014; Toyama et al., 2018).

Researchers have long attempted to increase the growth of plants by adding chemical compounds in addition to mineral fertilizers. In 1917, Bottomley reported that water extracts from bacterized peat contained certain growth-promoting organic compounds for *Lemna minor*, namely, auximones (Bottomley, 1917, 1920). Gorham clearly showed that sucrose at concentrations higher than 1% increased the growth of *Spirodela polyrhiza* (Gorham, 1945). Landolt found that sucrose promoted the growth of all *Lemnoideae* strains tested under low light intensities, and this effect was attenuated under higher intensities (Landolt, 1957). Lactic acid has been known to act as a plant growth regulator for carrot, marigold, sunflower and sugarcane (Hildebrandt et al., 1954). Further, dimers or larger polymers of L-lactic acid at 1,000 ppm were shown to promote the growth of both *L. minor* and *Zea may*s L. by 2-fold (Kinnersley et al., 1990). Organic fertilizer is now widely used in crop and vegetable farms.

Developing technologies that enable duckweed growth by the addition of chemical compounds requires the understanding of the basic traits of duckweeds exposed to exogenously added representative bioactive organic compounds. Plant growth regulator compounds, including plant hormones, profoundly influence the growth and differentiation of plant cells, tissues, and organs. Many biotic and abiotic chemical compounds are known to affect plants; for example, regulation of cell size and cell division by ethylene, regulation of cell cycle by auxins (IAA) and cytokines, induction of seed germination and stem elongation by gibberellins (GA), maintenance of seed dormancy by abscisic acid (ABA) and an endogenous signaling against pathogens and abiotic stresses by salicylic acid (SA) and jasmonic acid (JA).

In aquatic plants such as *L. minor*, the effects of growth regulators on biomass production have rarely been investigated. Gibberellic acid (GA_3) has been reported to homeopathically potentize and reduce the growth rate of *L. gibba* (Scherr et al., 2009). However, the reproducibility of the result was confirmed later by the same group, showing that growth was increased by GA_3 (Majewsky et al., 2014). Scherr et al. (2007) also found that eleven potentized compounds, including IAA, decreased the growth of *L. gibba*. Idris et al. (2007) prepared three mutants of plant growth-promoting *Bacillus amyloliquefaciens* FZB42 that showed reduction of 15–38% in the level of IAA. The growth-promoting activity of these mutants against *L. minor* concomitantly reduced to 17–19%, suggesting that IAA is a predominant plant growth-promoting factor. These controversial and confusing findings prompted us to determine comprehensively the effect of exogenous general plant growth regulator compounds on the growth of *L. minor*.

MATERIALS AND METHODS

Duckweed Culture

Sterile *L. minor* RDSC #5512 isolated from the Botanical Garden of Hokkaido University was used in this study. About 50 fronds of *L. minor* were aseptically transferred every 10 d to new 500 mL flasks containing 250 mL Hoagland medium for subculture. Hoagland medium contains per liter of 36.1 mg KNO_3, 293 mg K_2SO_4, 103 mg $MgSO_4$-$7H_2O$, 147 mg $CaCl_2$-$2H_2O$, 5 mg $NaHPO_4$-$2H_2O$, 0.95 mg H_3BO_3, 0.39 mg $MnCl_2$-$4H_2O$, 0.03 mg $CuSO_4$-$5H_2O$, 0.08 mg $ZnSO_4$-$7H_2O$, 0.23 mg H_2MoO_4, and 3.33 mg $FeSO_4$-$7H_2O$. The pH was adjusted to 7.0 using sodium hydroxide. Plant culture conditions were 28°C, 60–70% humidity, 5000 lux (75 $\mu mol/m^2/s$ photon density) and photoperiod of 16 h light / 8 h dark. Sterility of *L. minor* was confirmed when no bacterial colony was formed on R2A agar plate after incubation at 30°C for 3 d. R2A agar contained per liter 0.50 g each of yeast extract, proteose peptone (Difco no. 3), casamino acids, glucose, and soluble starch; 0.3 g each of Na-pyruvate and K_2HPO_4; and 0.05 g $MgSO_4$-7 H_2O. The pH was adjusted to 7.2 using sodium hydroxide. Agar was added at 15 g before autoclaving.

Plant Growth Regulators

The plant growth regulator compounds used in this study were GA_3 (Sigma Aldrich), indole-3-acetic acid (IAA; Wako Pure Chemical Ind. Ltd.), SA (Wako), 1-aminocyclopropane-1-carboxylic acid (ACC; Wako), aminoethoxyvinylglycine (AVG; Sigma Aldrich). Ascorbic acid (Wako) was also tested for the plant growth experiment. Stock solution of chemicals was prepared in MilliQ water and filter-sterilized.

Evaluation of the Growth of *L. minor*

Sterile *L. minor* was transferred to Petri dish containing appropriate amount of Hoagland medium, its two fronds were separated using a pair of forceps and inoculated into 100 mL flasks containing each compound in 50 mL Hoagland medium. Flasks were prepared in three replicates for every experiment. The effects of these compounds on plant growth were quantified by evaluating four parameters: frond numbers, fresh weight or dry weight, root length and chlorophyll $a+b$ content. After the number of fronds was counted, five plants of 10 d culture were randomly obtained from each flask, and the length of the longest roots was measured as the root length for every treatment. For the fresh weight, whole plants in the flask were carefully removed, blotted dry on paper towel, and weighed by a micro-scale (Sartorius, Göttingen Germany). Dry weight was measured in nylon tea bag filters after freeze drying (FDU-1110; EYELA).

Chlorophyll contents of *L. minor* were measured as previously described (Suzuki et al., 2014). Briefly, several plants were placed into 2 mL Eppendorf tubes containing 0.5 g of 0.1 mm glass beads (YGB01, Yasui Kikai, Japan). Next, 1 mL of cold ethanol previously saturated with $Ca(CO_3)_2$ was added and vigorously shaken by using a Multi-beads shocker MB755U(S) (Yasui Kikai) at 2,700 vibration per minute for 300 s at 4°C. After glass beads and cell debris were removed by centrifugation, the chlorophyll content was quantified by measuring photometric absorption at 649 and 665 nm. The concentration in units of μg/mL of chlorophyll *a* was calculated as [13.5275 (A_{665}) – 5.2007 (A_{649})] and chlorophyll *b* as [−7.0741 (A_{665}) + 22.4327 (A_{649})]. The chlorophyll content

was determined as mg chlorophyll *a*+*b*/g fresh weight of specimens. Statistical significance of each value was validated by unpaired t-test and one-way ANOVA using triplicate samples.

RESULTS AND DISCUSSION

GA_3

Rice farmers of Japan had long noted a fungal disease called "foolish seedling" or *bakanae* disease that causes rice plants to grow taller and reduces seed production. Plant pathologists found that this symptom in rice plants was caused by chemicals secreted by a pathogenic fungus, *Gibberella fujikuroi*. These fungal compounds possessing plant growth promoting activity were later named gibberellins A_1, A_2, and A_3 or gibberellic acid (Takahashi et al., 1955). GA_3 can also promote the growth of maize by inhibiting peroxidase secretion into the apoplast (Fry, 1979). The treatment of maize plants with GA_3 increased the length of the leaf blade elongation zone compared with that in the control via an increase in cell division and cell elongation duration (de Souza and MacAdam, 2001). In other plants such as potatoes as well, GA_3 treatments significantly increased the number of sprouts, stems, and tubers at very high concentrations of 100 mM in the cultivar "Fambo," but the total biomass did not increase (Virtanen et al., 2013). Oota and Tsudzuki (1971) reported that the number of fronds was increased to 125% by the application of 100 μM GA_3. Inada and Shimmen (2000) investigated the effects of the exogenous addition of GA_3 to *L. minor*. These researchers observed no significant changes in root length up to 1 μM, but found that it slight decrease at 10 μM concentration. Conversely, they also reported that exogenous addition of >1 mM GA_3 to the root segment of *L. minor* for 12 h inhibited root elongation in a concentration-dependent manner up to 1 μM (Inada and Shimmen, 2000). These researchers attributed this inconsistency to the fact that the root tip part was not included in the root segments used and the duration of exposure to GA_3 differed. However, they reported that uniconazole-P (Un-P), a gibberellin biosynthesis inhibitor, also inhibited the elongation of segments at 10 nM similar to the decrease in the root length in the experiment involving whole plants. These data show that the effect of GA_3 on the growth of *L. minor* is still controversial.

In our experiment, *L. minor* was grown in the presence of different concentrations of GA_3 ranging from 0 to 50 μM in Hoagland medium (**Figure 1**). After 10 d, the growth traits of *L. minor*, such as the frond number, root length, fresh weight, and chlorophyll content, were observed. Both the number of fronds and root length were not considerably different among the treatments, suggesting that *L. minor* is insensitive to exogenous GA_3 at these concentrations (**Figures 1A,C**). This result was also supported by the fresh weight of the plants (**Figure 1B**). Conversely, the chlorophyll content was increased from 0.37 to 0.59 mg g^{-1} fresh weight after treatment with 5 μM GA_3 (**Figure 1D**). On the other hand, we observed that ABA, another endonegenous stress signaling compound and an antagonist of GA severely inhibited the growth of *L. minor* (Supplementary Figure 1).

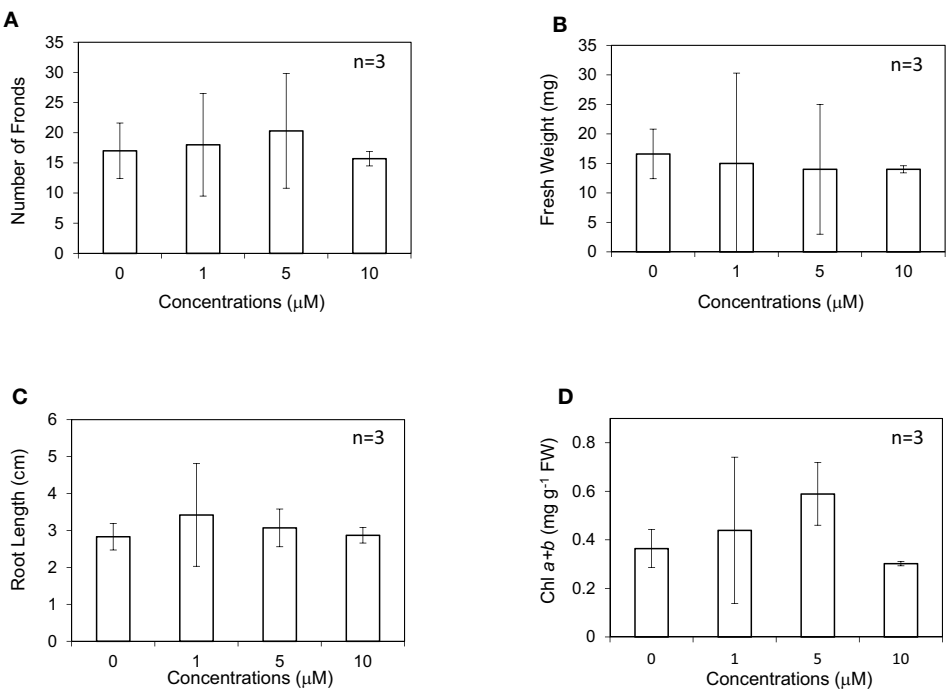

FIGURE 1 | Effect of gibberellic acid (GA_3) on the frond number **(A)**, fresh weight **(B)**, root length **(C)**, and chlorophyll content **(D)** of *Lemna minor.* Each value was measured after 10 d of cultivation. SD values are shown in line segments.

IAA

Indole-3-acetic acid is the principal auxin in plants; it controls many important physiological processes, including cell enlargement and division, tissue differentiation, and responses to light and gravity (Taiz and Zeiger, 1998). Auxins stimulate cell elongation and influence a host of other developmental responses such as root initiation, vascular differentiation, tropic responses, apical dominance, and the development of auxiliary buds, flowers and fruits. Auxins are synthesized in the stem and root apices and transported through the plant axis. Several other indole derivatives, as precursors to IAA, are known to express auxin activity, probably by converting to IAA in the tissue. This hormone is widely used in agriculture and horticulture to prevent leaf abscission and fruit drop, promote flowering and fruiting, and control weeds. Exogenous application of IAA has been reported to increase the growth of the root and shoot of wheat seedlings and to protect plants against stress (Egamberdieva, 2009). Yang et al. (1993) reported significant promotion of stem elongation in pea seedlings by exogenous IAA at concentrations from 50 to 1,000 μM. Conversely, exogenous IAA at 1 μM was found to retard germination and decrease the fresh weight of germinated soybean seeds. A small, but significant growth promotion by IAA up to 25 ppm (143 μM) was reported for crude culture of *L. minor* (Blackman and Robertson-Cuninghame, 1954); they also noted frond epinasty and root inhibition at higher concentrations. Conversely, Inada and Shimmen (2000) reported that exogenous IAA up to 1 μM inhibited the elongation of root segments in *L. minor*.

In this study, *L. minor* was grown in the medium containing different concentrations of IAA at 0–50 μM. The root length was found to decrease by approximately 50% at all the concentrations of IAA higher than 1 μM (**Figure 2C**; Inada and Shimmen, 2000). Conversely, frond numbers, fresh weight, and chlorophyll content of *L. minor* were not affected by the addition of IAA except for significant growth inhibition at 50 μM (**Figures 2A,B,D**). Notably, the amount of chlorophyll was not decreased even after treatment with 50 μM IAA. We also tested IAA at 0.1 and 0.5 μM but no plant growth-promoting activity was observed (data not shown). In contrast to the generally positive effects of IAA on the growth of soil plants, no plant growth-promoting activity was observed in our experiment for *L. minor*.

SA

Salicylic acid is an endogenous signaling compound abundantly produced under abiotic and biotic stresses, including drought, temperature, heavy metals, and pathogen infection. Despite its broad distribution in plants, SA has basal levels differing widely among species, with up to 100-fold differences been reported (Raskin et al., 1990). The role of SA in photosynthetic parameters and short-term acclimation to high light was deduced from the phenotypes shown by *Arabidopsis thaliana* plants with contrasting endogenous SA levels. Moreover, the effects of exogenous SA on photosynthesis parameters were found to differ depending on the dose and plant species tested. High concentrations (1–5 mM) of SA reduced the photosynthetic rate and RuBisCO activity in barley plants (Pancheva and Popova, 1998). *A. thaliana* mutants with different endogenous levels of SA showed growth phenotype that was inversely correlated to SA content (Vicente and Plasencia, 2011).

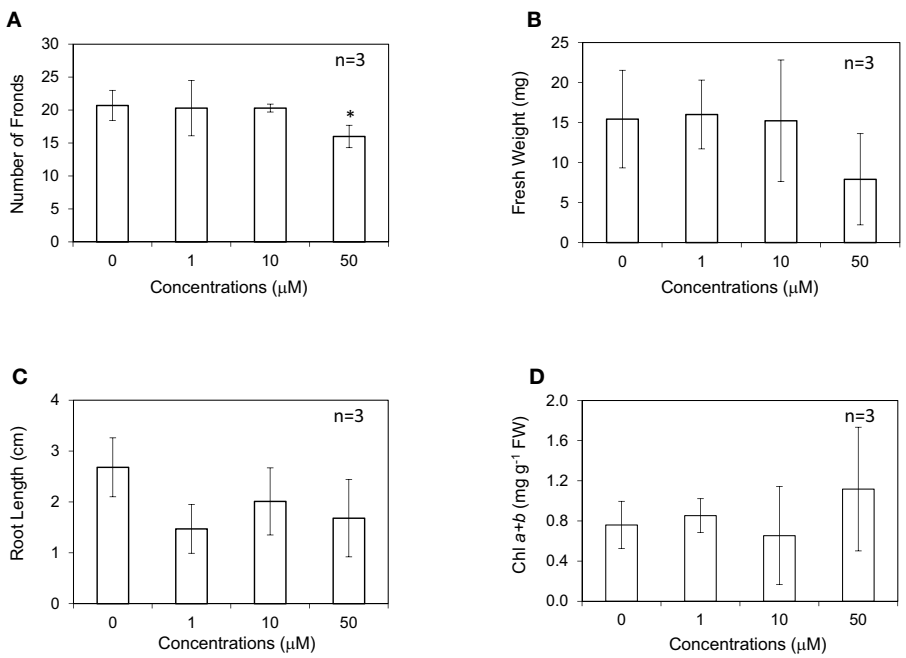

FIGURE 2 | Effect of indole-3-acetic acid (IAA) on the frond number **(A)**, fresh weight **(B)**, root length **(C)**, and chlorophyll content **(D)** of *Lemna minor.* Each value was measured after 10 d of cultivation. SD values are shown in line segments. *$p < 0.05$ against 0 μM control.

Shimakawa et al. (2012) reported that the amount of endogenous SA was increased under nutrient-poor conditions, and exogenous SA application induced flowering in *Lemna aequinoctialis*. This suggests that SA in *Lemnoideae* plants is also a signaling compound in response to environmental stresses. Recently, the exogenous application of 50 μM SA was reported to reduce the accumulation of Cd, leading to less toxicity (Lu et al., 2018). In this study, *L. minor* was grown

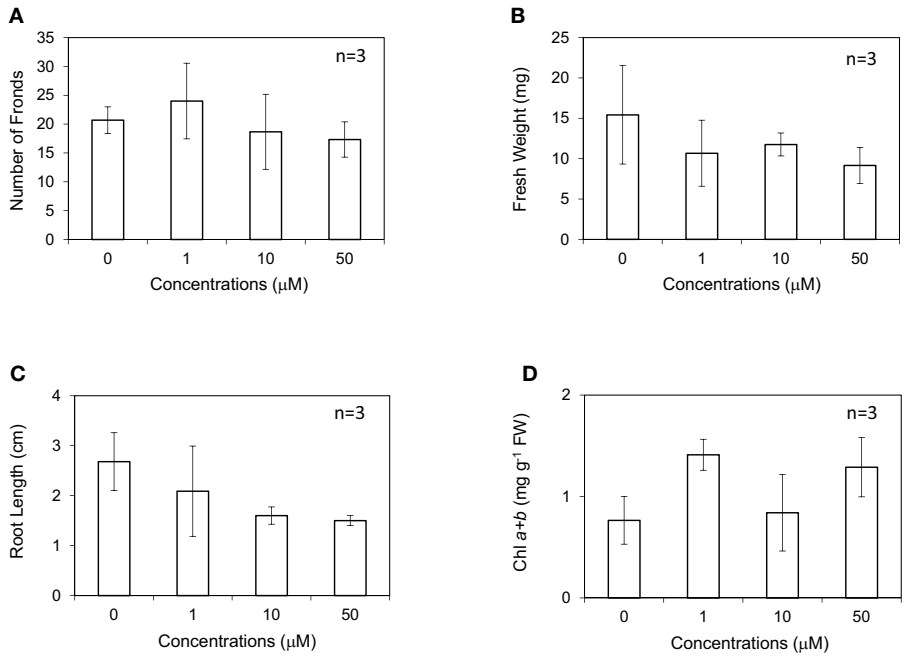

FIGURE 3 | Effect of salicylic acid (SA) on the frond number **(A)**, fresh weight **(B)**, root length **(C)**, and chlorophyll content **(D)** of *Lemna minor.* Each value was measured after 10 d of cultivation. SD values are shown in line segments.

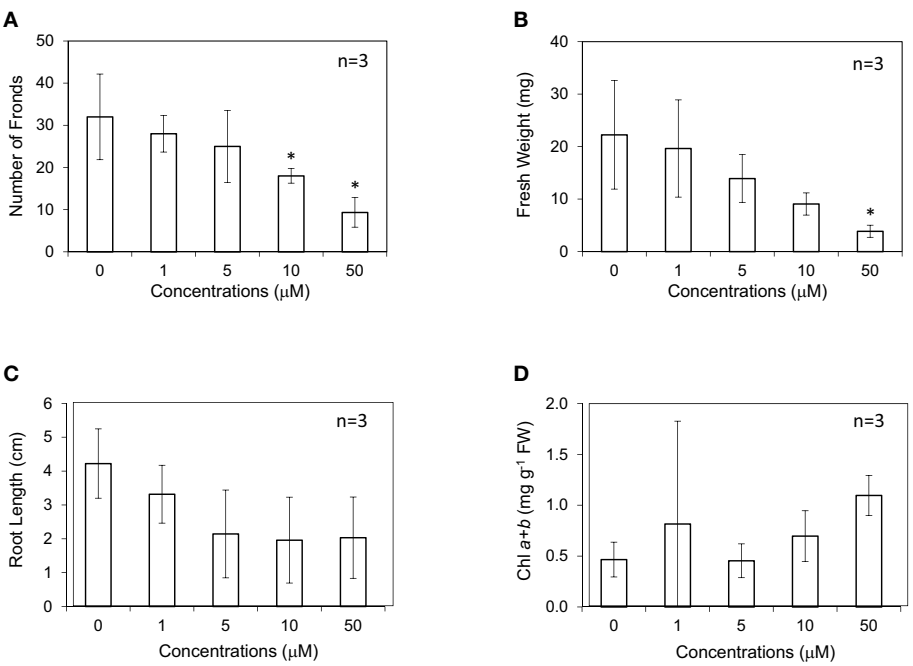

FIGURE 4 | Effect of 1-aminocyclopropane-1-carboxyl acid (ACC) on the frond number **(A)**, fresh weight **(B)**, root length **(C)**, and chlorophyll content **(D)** of *Lemna minor.* Each value was measured after 10 d of cultivation. SD values are shown in line segments. *$p < 0.05$ against 0 μM control.

in the medium containing different concentrations of SA at 0–50 μM. SA did not significantly affect the number of fronds, but generally and clearly decreased fresh weight and root length of *L. minor* (**Figures 3A–C**). Although the range of errors is large, the amount of chlorophyll was not found to decrease (**Figure 3D**). Another endogenous stress signaling compound, JA clearly inhibited the growth of *L. minor* (Supplementary Figure 1).

ACC

Ethylene affects plant growth at all stages of development from seed germination to organ senescence and promotion of fruit ripening (Corbineau et al., 2004). Ethylene is known to be a key initiator of fast underwater elongation of submerged plants (Jackson, 2008). Treatments of submerged and non-submerged *Oryza sativa* with ethylene stimulated internode elongation (Métraux and Kende, 1983). Moreover, when ethylene synthesis was blocked with aminooxyacetic acid and aminoethoxyvinylglycine (AVG) in partially submerged plants, internode elongation was inhibited. This growth inhibition was reversed when ethylene biosynthesis was restored with ACC, an immediate precursor of ethylene. ABA and ethylene are known to have antagonistic functions for controlling plant growth and development, including seed germination and early seedling development (Cheng et al., 2009). External addition of ACC to plants changes the convergent point between these two signaling pathways. Under light condition, *A. thaliana* shows an elongated hypocotyl and shortened root growth in the presence of 1 μM exogenous ACC (Smalle et al., 1997). When grown in darkness, both the hypocotyls and roots were reduced in the wild type at 0.5 μM and in the *are2* mutant at 10 μM of exogenous ACC (Vanderstraeten and Straeten, 2017).

Ethylene is known to be a gibbosity regulator of *L. gibba* and *L. minor* (Elzenga et al., 1980); however, its effect on growth has not been investigated. In this study, *L. minor* was grown with different concentrations of ACC under 16-h light photoperiod condition. In contrast to submerged plants, the effects of exogenous ACC on *L. minor* growth were apparently negative (**Figures 4A–C**). Addition of 1–50 μM of ACC significantly decreased the frond numbers, fresh weight, and root length. This might be explained by the effect of enhanced ethylene production in the plant body that reduced the growth and development. With regard to the effect of ACC on chlorophyll content, although the range of errors was large, the amount of chlorophyll was surprisingly increased up to 50 μM ACC (**Figure 4D**).

AVG

AVG is often used as a specific inhibitor of ethylene biosynthesis to analyze the effects of ethylene on plant growth, development and stress response (Abeles et al., 1992) that potentially can mitigate growth suppression under stress condition. Further, the addition of 1 μM of AVG was found to significantly suppress the pistil length of *L. gibba* (Mader, 2004) but no report about the effect of AVG on overall plant growth are yet available.

AVG remarkably inhibited the growth of *L. minor*. AVG decreased the frond number, fresh weight and chlorophyll content of *L. minor* at all concentrations (**Figures 5A–D**). Roots were lost in almost all the surviving plants. Growth of *L. minor* was also impaired by the addition of very low concentration (0.5 μM) of AVG (data not shown). Interestingly, the amount of chlorophyll was decreased, but not as much as the degree of growth defect (**Figure 5D**). Only this trait can be rationally explained by the inverse effect of ACC and AVG on the biosynthesis of ethylene. However, our experimental results indicate that AVG is not a compound useful for enhanced production of duckweed biomass. Notably, spraying of AVG onto the shoots of cotton blocked ethylene accumulation in the leaves under waterlogging stress. This application subsequently improved leaf growth and enhanced the production of both fruit and cotton cultivars even under non-waterlogging condition (Najeeb et al., 2015).

AsA

L-Ascorbic acid (AsA) is an abundant water-soluble antioxidant in plants that is capable of scavenging toxic reactive oxygen species (ROS) emitted during photosynthesis. Overproduction of ROS is triggered by various environmental stress-induced oxidative damages. Exogenous AsA is known to protect lipids and proteins against oxidative stresses, thereby promoting the growth of plants. Application of AsA to foliar has been reported to improve the growth of strawberry plants under high temperature condition (44°C; Ergin et al., 2014). For example, exogenous AsA at 3 mM increased cell turgidity of the strawberry plants and hence their growth. Foliar or seed treatment with 20 and 40 mg L^{-1} AsA improved the seedling growth and yield of maize under low temperature stress (Ahmad et al., 2014). Moreover, exogenous application of AsA has been found effective in mitigating the adverse effects of water stress in wheat (Hafez and Gharib, 2016). Once used, AsA can be recycled by several different mechanisms in the plant cells (Gallie, 2013; Akram et al., 2017).

AsA showed significant growth-promoting activity in *L. minor*. The highest increase in frond number was observed at 10 ppm, 57 μM AsA, which was 1.7-fold higher than that in the control experiment with no AsA addition (**Figures 6A,B**). The number of fronds was slightly lower at 50 ppm AsA than at 10 and 5 ppm AsA; however, the dry weight of plants was equivalent or even slightly higher (**Figure 6C**). All fronds of *L. minor* grown in the presence of AsA showed higher chlorophyll content than that in the control, suggesting that AsA reduced ROS accumulation in the apoplast. The chlorophyll content after treatment with 10 ppm AsA was higher than that in the control (**Figure 6D**). Recently, Ishizawa et al. (2017) reported that *L. minor* accumulated ROS and expressed an evident antioxidant enzyme activity. Furthermore, the antioxidant enzyme activity was markedly increased in the presence of plant growth-inhibiting bacteria. These results are not inconsistent with our observation that an antioxidant AsA increased the growth of *L. minor*.

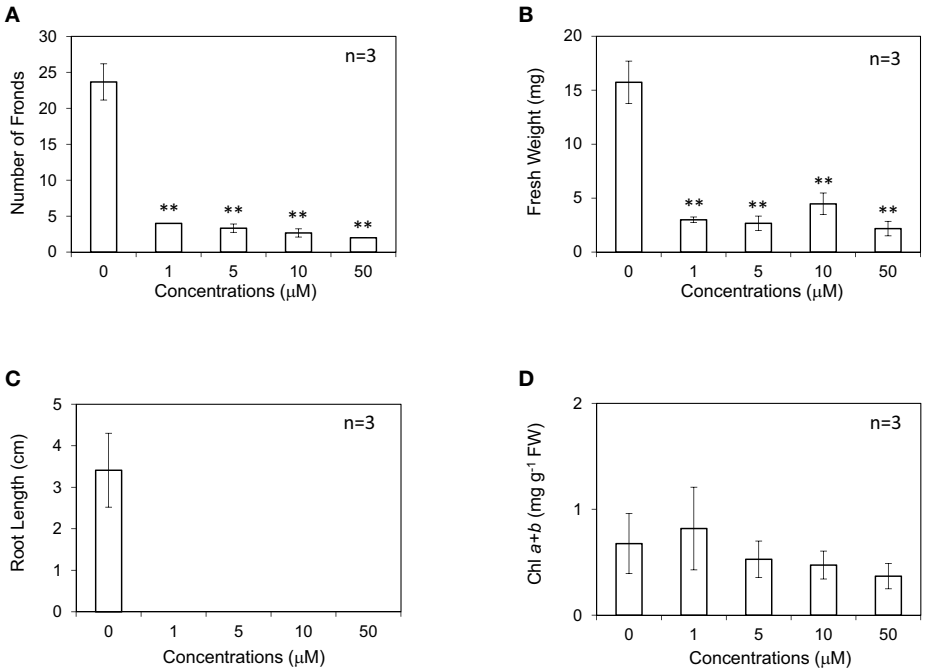

FIGURE 5 | Effect of aminoethoxyvinylglycine (AVG) on the frond number **(A)**, fresh weight **(B)**, root length **(C)**, and chlorophyll content **(D)** of *Lemna minor.* Each value was measured after 10 d of cultivation. SD values are shown in line segments. $^{**}p < 0.01$ against 0 μM control.

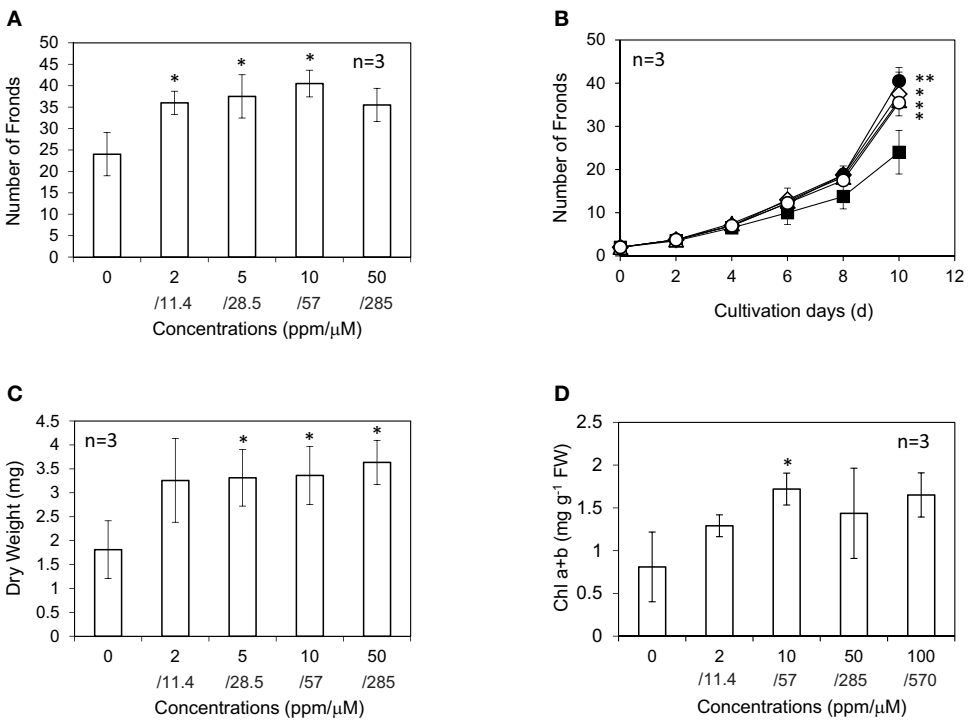

FIGURE 6 | Effect of ascorbic acid on the frond number **(A)**, frond number during 10 d **(B)**, dry weight **(C)**, and chlorophyll content **(D)** of *Lemna minor.* Each value was measured after 10 d of cultivation except for **(B)**. Symbols are 0 ppm, closed square; 2 ppm, open triangle; 5 ppm, open diamond; 10 ppm, closed circle; 50 ppm, open circle. SD values are shown in line segments. $^{*}p < 0.05$ against 0 μM control. $^{**}p < 0.01$ against 0 μM control.

CONCLUSIONS

- Exogenous application of the plant hormones gibberellic acid (GA_3), indole-3-acetic acid (IAA), and salicylic acid (SA) showed no apparent growth-promoting effect on frond number and fresh weight of *L. minor*. Root length was tended to be decreased by IAA and SA. Jasmonic acid (JA) moderately and abscidic acid (ABA) severely inhibited the growth of *L. minor*.
- Growth of *L. minor* was inhibited by both antagonistically functioning compounds in ethylene production, ACC and AVG, where the latter acted more severely. These results suggest that *L. minor*, a *Lemnoideae* plant, behaves uniquely against plant growth regulator compounds.
- AsA significantly promoted the growth of *L. minor* under normal growth condition at concentrations higher than 2 ppm (11.4 μM). To the best of our knowledge, AsA has till date not been shown to promote the growth of *Lemnoideae* plants.

AUTHOR CONTRIBUTIONS

DU, AK, RJ, and MS designed and performed the experiments and drafted the manuscript. KM and MM interpreted the results revised the manuscript and supervised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00251/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Structural and Biochemical Properties of Duckweed Surface Cuticle

Nikolai Borisjuk[1], Anton A. Peterson[1], Jiyang Lv[2], Guorun Qu[2], Qian Luo[2], Lei Shi[2], Guimin Chen[1], Olena Kishchenko[1], Yuzhen Zhou[1] and Jianxin Shi[2]**

[1] *Jiangsu Key Laboratory for Eco-Agricultural Biotechnology around Hongze Lake, School of Life Science, Huaiyin Normal University, Huaian, China,* [2] *Joint International Research Laboratory of Metabolic and Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China*

The plant cuticle, which consists of cutin and waxes, forms a hydrophobic coating covering the aerial surfaces of all plants. It acts as an interface between plants and their surrounding environment whilst also protecting them against biotic and abiotic stresses. In this research, we have investigated the biodiversity and cuticle properties of aquatic plant duckweed, using samples isolated from four different locations around Hongze lake in Jiangsu province, China. The samples were genotyped using two chloroplast markers and nuclear ribosomal DNA markers, which revealed them as ecotypes of the larger duckweed, *Spirodela polyrhiza*. Duckweed cuticle properties were investigated by compositional analysis using Gas Chromatography coupled with Mass Spectroscopy (GC-MS) Flame Ionization Detector (GC-FID), and ultrastructural observation by cryo-Scanning Electron Microscopy (cryo-SEM). Cuticle compositional analysis indicated that fatty acids and primary alcohols, the two typical constituents found in many land plant cuticle, are the major duckweed wax components. A large portion of the duckweed wax fraction is composed of phytosterols, represented by campesterol, stigmasterol, sitosterol and their common precursor squalene. The cryo-SEM observation uncovered significant differences between the surface structures of the top air-facing and bottom water-facing sides of the plant fronds. The top side of the fronds, containing multiple stomata complexes, appeared to be represented by a rather flat waxy film sporadically covered with wax crystals. Underneath the waxy film was detected a barely distinguished nanoridge net, which became distinctly noticeable after chloroform treatment. On the bottom side of the fronds, the large epidermal cells were covered by the well-structured net, whose sections became narrower and sharper under cryo-SEM following chloroform treatment. These structural differences between the abaxial and adaxial sides of the fronds evidently relate to their distinct physiological roles in interacting with the contrasting environments of sunlight/air and nutrients/water. The unique structural and biochemical features of *Spirodela* frond surfaces with their rapid reproductive cycle and readily availability genome sequence, make duckweed an attractive monocot model for studying the fundamental processes related to plant protection against ultraviolet irradiation, pathogens and other environmental stresses.

Keywords: duckweed, *Spirodela polyrhiza*, biodiversity, genotyping, cuticle wax, fatty acids, phytosterols

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Reviewed by:
Uwe Heinig,
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University of Western Ontario, Canada

****Correspondence:***
Nikolai Borisjuk
nborisjuk@hytc.edu.cn
Jianxin Shi
jianxin.shi@sjtu.edu.cn

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INTRODUCTION

The plant cuticle, a skin of the plant, forms a hydrophobic coating that covers aerial surfaces of all plants. In conjunction with stomata, the cuticle acts as an interface between plants and their surrounding environment, protecting them against a variety of abiotic stresses and pathogens. The cuticle not only acts as a protective interface but also controls the diffusion of molecules, with the most important function of the cuticle in protecting the aerial parts of terrestrial plants against water loss (Samuels et al., 2008).

Plant cuticle is generally composed of a cutin matrix covered or/and embedded with layers of cuticular waxes, however considerable variation in cuticle composition exists between species, plant organs and developmental stages (Yeats and Rose, 2013). The typical cutin matrix is represented by hydroxy and/or epoxy C16/C18 fatty acids cross-linked by ester bonds into an elastic polyester structure (Fich et al., 2016; Bakan and Marion, 2017). The waxes consist of a combination of various aliphatic molecules, such as very long fatty acids (with a chain length of C20-C24), primary alcohols (C22-C40), alkanes (C21-C35), aldehydes (C24-C36), ketones (C21-C35), and diketones (C22-C36). These lipid derivatives can be embedded in the cutin matrix (intracuticular waxes), or deposited on the cuticle surface (epicuticular waxes). These aliphatic waxes define one of the essential features of primary plant surfaces, their hydrophobicity. This serves to repel water, other aqueous solutions and small organisms (Müller and Riederer, 2005). Epicuticular waxes deposited on the outer cuticle surface often form crystals of different shape visible under an electron microscope (Barthlott et al., 1998). In addition to waxes, some other components like phenolic represented by cinnamic acid or flavonoids could be also present in the cutin matrix (Domínguez et al., 2011).

The biochemical composition, as well as biosynthesis pathways leading to the accumulation of cuticle components, have been intensively studied in a number of terrestrial plants. Most intensively in the model plant Arabidopsis, tomato, maize and barley (Jetter et al., 2007; Bernard and Joubes, 2013; Borisjuk et al., 2014). As a result of these efforts, substantial progress has been made in recent decades for characterizing the biochemical and molecular mechanisms of cutin and wax synthesis and export (Samuels et al., 2008; Kunst and Samuels, 2009), cutin monomer synthesis and assembly (Beisson et al., 2012), cuticle involvement in biotic and abiotic stress responses (Bessire et al., 2007; Seo and Park, 2011) and regulation of organ development (Ingram and Nawrath, 2017). However, many aspects of cuticle biology remain unclear, especially for specialized groups of plants, such as aquatic inhabitants. While aquatic lotus, *Nelumbo nucifera*, has been intensively studied in respect of the unique hydrophobic properties of its leaf surface (Ensikat et al., 2011), not much is known about the specificity of cuticle surface structure and biochemical composition in relation to inhabiting water surfaces.

Duckweed is a group of monocot aquatic plants endemic to most parts of the world. Its productivity can reach 80–100 tons dry mass per hectare per year, over 5 times as high as maize (Lam et al., 2014). In the process of biomass accumulation, duckweeds can very efficiently remediate different types of wastewater (Ziegler et al., 2016; Zhou et al., 2018). These complementary features - water remediation and fast biomass accumulation have made duckweed an object of intense global research interest in recent years (Lam et al., 2014; Appenroth et al., 2015). This increased level of research has resulted in the genome sequencing of two representative species *Spirodela polyrhiza* and Lemna minor (Wang et al., 2014; Van Hoeck et al., 2015; Michael et al., 2017), the establishment of specialized international conferences and an active and diverse academic community studying duckweed biology.

For aquatic plants, such as duckweed, which have an unlimited supply of water through constant contact of leaf and/or roots with water, water conservation inside the tissues should not be as important as it is for terrestrial plants grown under limited water supply. Instead, since duckweed has no option (mechanism) to protectively adjust frond position relative to sunlight, as terrestrial plants can do turning their leaves and flowers to an optimal position relative to the sun, the duckweed's frond surface should provide a sustainable protection against potentially harmful ultraviolet (UV) radiation. Protection against UV has been attributed mainly to the light scattering by the surface cuticular waxes (Long et al., 2003) and UV attenuation properties of various phenolic compounds incorporated into cuticle matrix (Rozema et al., 2002; Chen et al., 2013).

The second important function of the cuticle in aquatic plants concerns protection against pathogens, both airborne and those inhabiting the water environment. The interaction of aerial surfaces of land plants with microbial pathogens and insects has been intensively investigated, especially in the model plant Arabidopsis and some agriculturally important cereals, (Reina-Pinto and Yephremov, 2009; Serrano et al., 2014; Kumar et al., 2016). However, almost no studies exist on the role of cuticles in aquatic plant-pathogen interaction at water surfaces.

As duckweed is becoming a popular prospective source of biomass, its cuticles also define the plants nutrient quality and other industrial applications (Petit et al., 2017). In this study, for the first time we present data on the surface structure (SEM) and biochemical composition (GC-MS/GC-FID) of cuticle in great duckweed, *Spirodella polyrhiza*, a duckweed species with a great potential for wastewater remediation (Ziegler et al., 2016), production of biofuel and animals/fish feed (Cheng and Stomp, 2009).

MATERIALS AND METHODS

Plant Material

Four ecotypes of duckweed used in this study were collected at different locations around Hongze lake in Jiangsu province, China, in October 2016. The GPS coordinates for the samples collection are: N 33″38′41/E 118″58′33 for sample G (RDSC clone registration: 5545); sample K: N 33″22′43; E 118″53′23 (RDSC clone registration: 5546); sample M: N 33″19′41; E 118″51′43 (RDSC clone registration: 5547); sample N: N 33″17′40; E 118″49′45 (RDSC clone registration: 5548).

Identity Recognition

Prior to further examinations, duckweed samples were washed with water and cultivated on the surface of sterilized SH medium for 2 weeks. The identity of the collected duckweed ecotypes was examined by DNA barcoding using primers specific for chloroplast intergenic spacers *atpF-atpH* (ATP) and *psbK-psbL* (PSB) as previously described (Borisjuk et al., 2015). Additionally, the biodiversity of the collected duckweed strains was estimated by comparison of DNA sequences of intergenic spacers on the nuclear 5S ribosomal genes (rDNA). The DNA fragments of 5S rDNA spacers were amplified by PCR using 5S gene-specific primers DW-5S-F: CTTGGGCGAGAGTAGTACTAGG and DW-5S-R: CACGCTTAACTTCGGAGTTCTG, purified by gel electrophoresis and sequenced using the DW-5S-F primer. The obtained sequences were aligned using the "Online Analysis Tools" package (http://molbiol-tools.ca).

Microscopic Observation of Duckweed Surfaces

The air and water-facing surfaces of duckweed fronds and turions were examined using a cryo-scanning electron microscope (cryo-SEM, Hitachi S3400II). The fresh duckweed fronds and turions were carefully mounted on the copper stage with glue, immediately frozen with liquid nitrogen, sprayed with gold, and observed under low vacuum mode. Images were taken using its carrying camera.

The autofluorescence of duckweed surfaces was monitored using fronds decolorized by repeated incubation in 70% ethyl alcohol (Vitha et al., 1995) supplemented with 10% sucrose at 37°C. Following decolorization, a half of the fronds were incubated overnight at 37°C in 0.5M NaOH in order to remove cell wall bound phenolic acids, a potential source of autofluorescence (Ride and Pearce, 1979). The discolored fronds were sliced using LEICA CM1850 cryomicrotome. The autofluorescence of the resulted 20 μm slices, was observed using LEICA DM2500 fluorescent microscope equipped with 360 and 480 nm wavelength excitation filters. The phloroglucinol-HCl staining was performed following the protocol described by Donaldson and Williams (2018) with some modifications.

Identification and Quantification of Duckweed Waxes and Cutin Monomers

For wax analyses, lyophilized or fresh duckweed fronds were immersed in 2 ml chloroform using two time regimes. First, the fronds were treated with chloroform for 60 s twice and the two extracts were pooled; second, the fronds were treated with chloroform for 30 s. Following wax extraction, 50 μl of internal standard (C24 alkane; 10 mg/ 50 ml) was added. Extracts with internal standard were dried under a stream of nitrogen to a final volume of 100 μl. Next, 20 μl pyridine and 20 μl BSTFA [*N*,*O*-bis(trimethylsilyl) trifluoroacetamide] were added and the mixtures were incubated at 70°C for 40 min. Wax solutions were transferred to GC vials and analyzed by Gas Chromatography-Mass Spectrometry/Gas Chromatography-Flame Ionization Detector (GC-MS/GC-FID) as described by Zhu et al. (2013). For cutin analysis, samples that had been used in the wax extraction were exhaustedly extracted with chloroform/methanol (1:1 v/v) for 2 weeks with daily changing in the solvent. The remaining delipidated samples were transesterified with 1 mL of 1 N methanolic HCl for 2 h at 80°C. After the addition of 2 ml of saturated NaCl and 20 mg of dotriacontane (Fluka) as an internal standard, the hydrophobic monomers were subsequently extracted three times with 1 ml of hexane. The organic phases were combined, evaporated, and derivatized as described above. The GC-MS and GC-FID analysis were performed using a procedure identical to that of the wax analysis. Results were calculated as per unit surface area.

Statistical Analysis

Not less than four biological replicas were analyzed by GS-MS for each duckweed ecotypes. For each of the identified individual chemical substances the mean values and standard deviations were calculated. Surface areas of fronds samples were measured using ImageJ graphical software (https://imagej.nih.gov/ij/). Percentages of the individual substances in cuticle waxes and individual monomers in depolymerized cutin fraction were calculated using data of individual substances mean content in ug/cm^2 of duckweed fronds surface.

One-way multivariate analysis of variance (MANOVA) was performed using General Linear Model Multivariate analysis module of the "SPSS Statistics" (IBM) software. Statistical significances of effects of ecotypes variations on the values of individual substance content in wax and cutin were calculated using *Post Hoc* Multiple Comparisons Tukey's Tests. The results of this test include grouping of ecotypes into homogeneous subsets according to the calculated statistical significances of differences in each substance mean content.

Representative cryo SEM images were chosen from not less than 30 micrographs of individual fronds or turions of each ecotype.

RESULTS

The Identity of the Duckweed Strains

Four duckweed strains collected around Hongze lake, used in the experiments (**Figure 1**), were genotyped using dual chloroplast DNA barcodes (Borisjuk et al., 2015), atpF-atpH (ATP) and psbK-psbL (PSB) spacer sequences, and nuclear 5S ribosomal DNA (rDNA) spacer. Chloroplast DNA fragments, amplified by PCR, were directly sequenced using one of the amplification primers and blasted against the sequences available in the NCBI DNA database. Both ATP and PSB clearly identified the samples as large duckweed, *Spirodela polyrhiza* (Appenroth et al., 2013). The PSB sequences show no variations between the four *Spirodela* strains, while the ATP demonstrated some sequence variations between the isolates, with most of them being G/A substitutions (Supplement Figure 1). Sequence alignments of the 5S rDNA spacer, only allowed analysis of the variations between the isolates, as no similar sequences were found in the DNA database. The alignment revealed a short 6 nucleotide deletion in strain M, and a G to C substitution in strain K (**Figure 1B**).

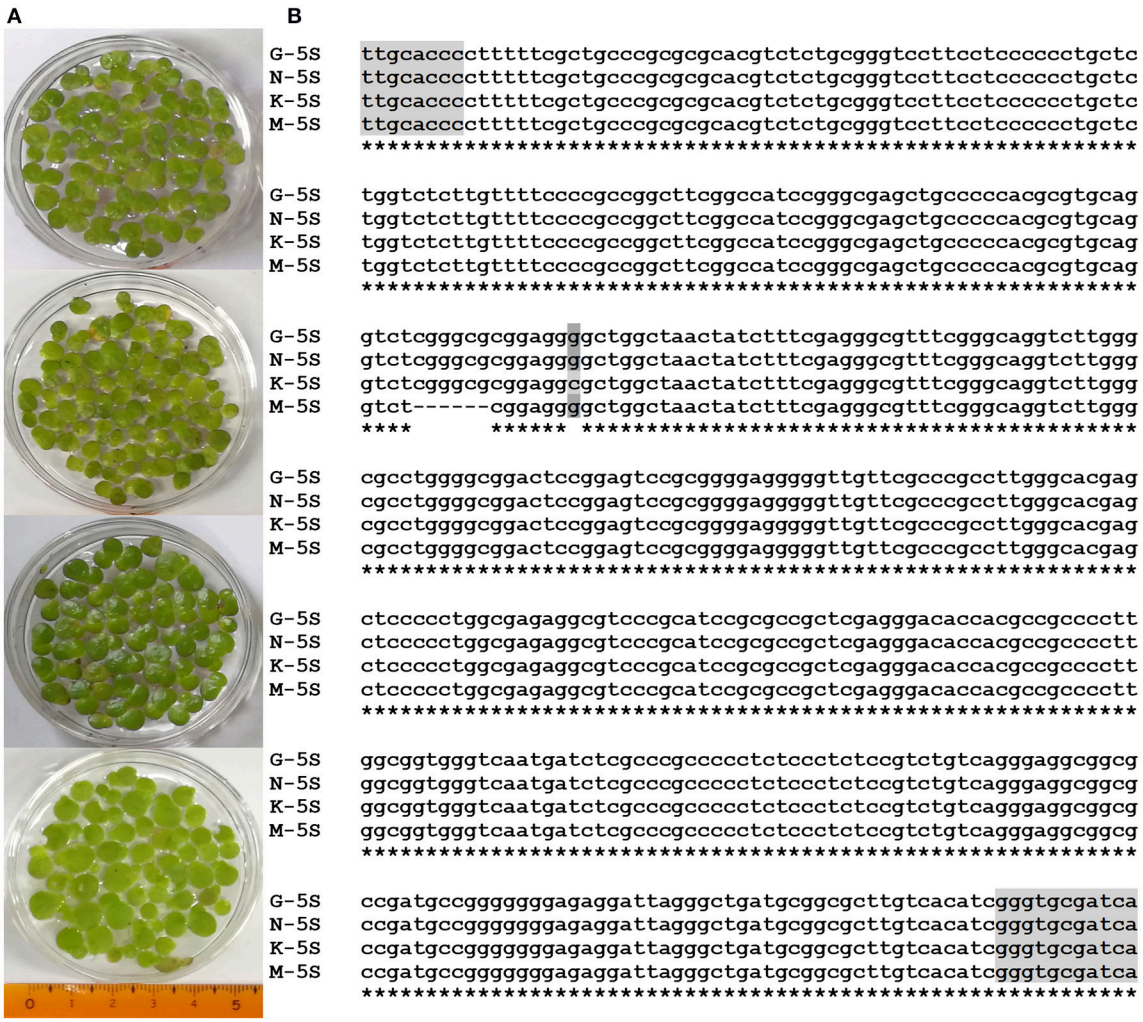

FIGURE 1 | Images of *S. polyrhiza* frond ecotypes G, K, M, and N **(A)**, and sequence alignment of their 5S rDNA spacers **(B)**. Parts of sequences encoding 5S ribosomal RNA are highlighted by gray.

Cryo-SEM Images of Duckweed Cuticle Surface

To investigate the structural features of the frond and turion surfaces we have performed cryo-SEM analysis, focusing on both intact surfaces of duckweed fronds and turions and the frond surfaces treated with chloroform (to remove epicuticular waxes). The air-facing and water-facing surfaces of all four isolates of *S. polyrhiza* were examined and the representative images of ecotypes N and M are presented in **Figure 2**.

The intact adaxial, air-facing side of frond cuticle of the investigated duckweed ecotypes, appeared as a predominantly smooth film spread with distinct stomata and some irregular unevenness, covering most of the morphology of underlying epidermal cells (**Figures 2A**,**B**). On the surface of this film, solitary wax crystalloids in the form of "granules" could be observed to be sparsely deposited as proposed by Barthlott et al. (1998) (**Figures 2A**,**B**). It was noted that ecotype N (**Figure 2B**) has a higher density of surface wax granules deposition, compared to ecotype M (**Figure 2A**). After being treated with chloroform, the wax "granules" and the waxy film were removed and this chloroform treatment transformed the original smooth waxy surface into the net-like nanoridge surface (**Figures 2C**,**D**).

The abaxial, water-facing side of the fronds showed a significantly different surface structure from the adaxial side. It did not have stomata (**Figure 2E**) but had well organized large oval cells (with the size of about 40 μm) fully covered with a thick waxy film, on which an extracellular net (with a single section diameter of 20–30 μm) formed. Upon chloroform treatment, the waxy film was removed, while the extracellular nanoridge became thinner and the topography of epidermal cells appeared, which seemed to have lost their rigidity (**Figure 2F**).

Similar to fronds, the *Spirodela* turions, displayed a very distinct surface structure of abaxial and adaxial sides (**Figure 3**). The abaxial turion side, defined by the absence of stomata, had a structure to a large extent resembling that of the frond's abaxial side (similar size of cells, ∼40 μm and sections

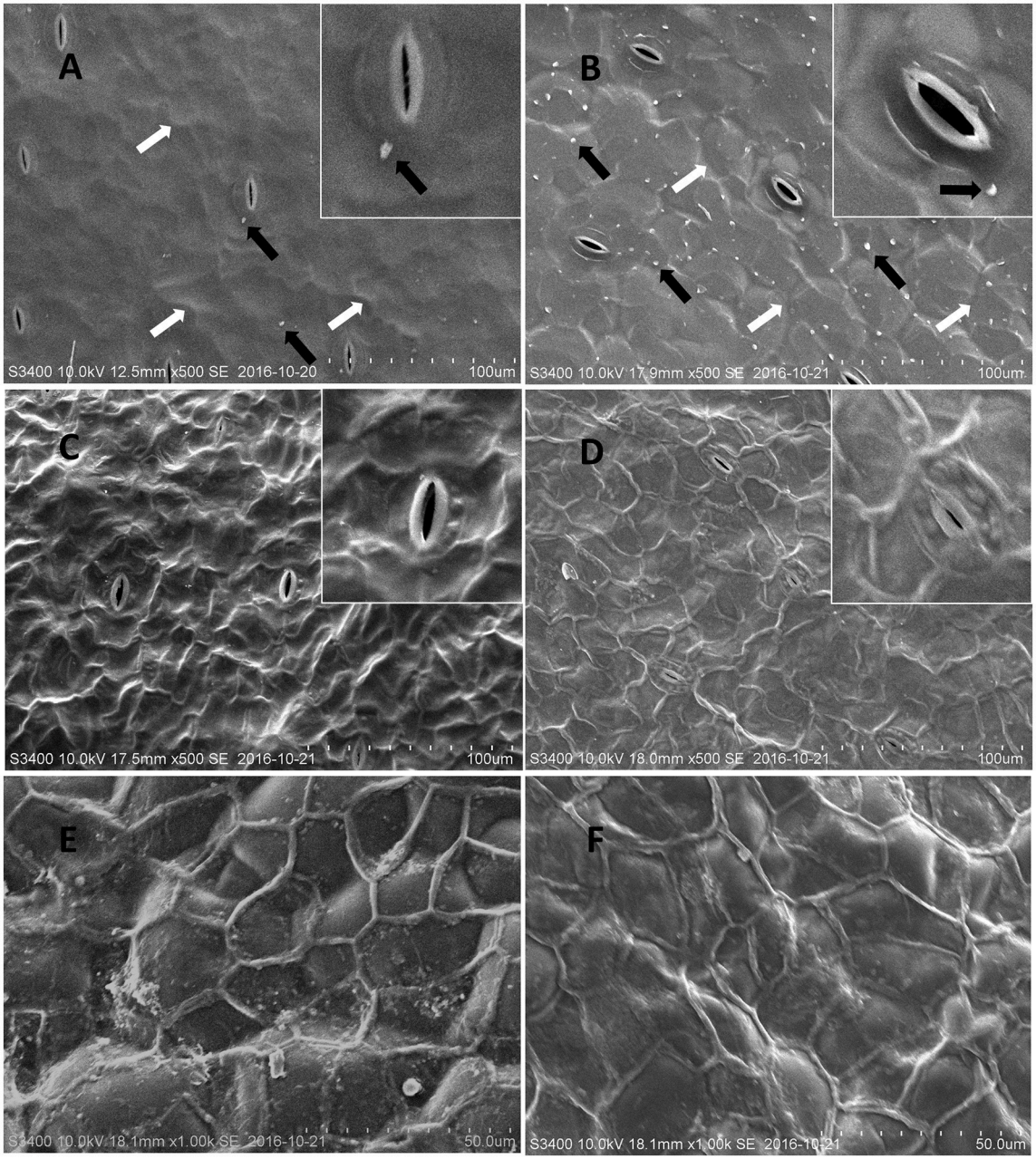

FIGURE 2 | Representative cryo-SEM micrographs of *S. polyrhiza* frond surfaces. Images of the intact adaxial (air-facing) side of *S. polyrhiza* fronds from strain M **(A)** and strain N **(B)**. Images of the adaxial side of *S. polyrhiza* fronds following treatment with chloroform from strain M **(C)** and strain N **(D)**. Images of the intact **(E)** and chloroform treated **(F)** abaxial (water-facing) side of *S. polyrhiza* frond from strain M. White arrows indicate unevenness in areas of epidermal cells joints and black arrows indicate wax crystalloids.

of net, ∼20–30 μm), with a more profoundly expressed extracellular net (**Figure 3B**). Whereas, the adaxial side of turions, was distinguished by well-defined stomata complexes with closed stomata and did not have the similar smooth waxy surface appearance to that of the frond's abaxial side. The contours of the epidermal cells, with the size of about 20 μm, were very well visible with the clearly defined borders between them. Few structures, similar to wax "granules" on the fronds surface could be detected on the dense cell surfaces.

Composition of the Surface Waxes of Fronds and Turions of *Spirodela polyrhiza*

The composition of waxes on the fronds and turions of four ecotypes (G, K, M, and N) of *Spirodela polyrhiza* was

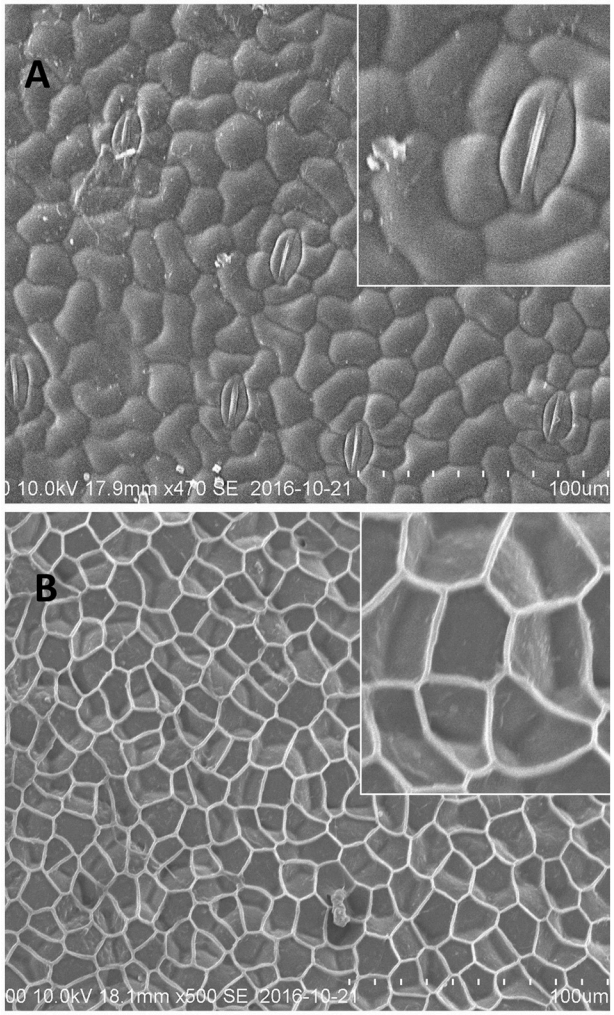

FIGURE 3 | Representative cryo-SEM micrographs of *S. polyrhiza* turion surfaces. **(A)** Adaxial side of the turion with stomatal complexes. **(B)** Abaxial side of the turion.

qualitatively and quantitatively analyzed using GC-MS and GC-FID, respectively. The wax fraction extracted from the fronds for 2 minute resulted in a mixture of free fatty acids, esters, fatty alcohols, alkanes, and phytosterols. Their compositions are shown in **Figure 4A**. Surprisingly, free fatty acids dominated, accounting for approximately 60 to 70% of the total soluble fraction; the next most common was phytosterols, accounting for about 20% of the total. Composition details for the major compounds is presented in **Figure 4B**. Repeating the procedure with an extraction time of 0.5 min revealed a similar pattern in major components, but the amounts differed. The percentage of free fatty acids became 18.8%, replaced by phytosterols, which increased to about 60% (Supplement Figure 2). The free fatty acids included both saturated and unsaturated types, ranging from 16 to 24 carbon atoms in length. When extracted for the longer period, the major individual fatty acids were saturated C16:0 (palmitic acid) and unsaturated C18:3 (α-linolenic acid), which is rather characteristic for intracellular membrane lipids, but at the shorter period, they were common cuticular waxes, saturated C16:0, C18:0, and C24:0.

Among the phytosterols was stigmasterol, campersterol, squalene, and β-sitosterol, the first representing the major component.

The saturated alcohols ranged in carbon chain length from 20 to 26, but they were predominantly 22 to 24 atoms in length, no matter the extraction time.

The wax composition of turions was analyzed for *S. polyrhiza* acotype N, which was also used to characterize the turion surface by SEM (**Figure 3**). The major component of this wax fraction is fatty acids clearly dominated by saturated C24:0 (**Figure 5**). The wax of turions also contains saturated fatty alcohols, predominantly those 22 to 24 carbon atoms in length, and esters and phytosterols in lesser amounts.

Biochemical Analysis of Cutin in Duckweed Fronds and Turions

The cutin monomers found in duckweed fronds included several subgroups of fatty acids and aromatic acids as well as one with a phenolic ring–cinnamic acid (**Figure 4C**). In total, the fatty acids comprised more than 95% of the cutin monomers. Composition details of the major individual monomers are presented in **Figure 4D**.

The major individual fatty acids were saturated C16:0 (palmitic acid), comprising about 20% of the total soluble waxes, saturated C24:0, saturated 2-hydroxylated C24:0 acids, and unsaturated C18:2 (linoleic acid), each comprising about 8% of the total, and unsaturated C18:3, comprising about 35% of the total. The pattern of cutin monomers in the fronds was similar to that found in the cutin extracted from turions of ecotype N, but cutin from the turions contain a significantly higher percentage of C18:2 and less C18:3 compared to the fronds (**Figures 5C, D**).

Because the presence of cinnamic acid, a common aromatic monomer of the polyaromatic domain of suberin and lignin (Bernards et al., 1995), could indicate suberinized layers in the duckweed epidermal cell walls, we investigated this possibility by performing autofluorescence tests on the ethanol-decolorized fronds. This treatment also removes flavonoids (Ferreira and Pinlio, 2012), the primary fluorescent agents in plant cuticle (Donaldson and Williams, 2018). As previously shown (Donaldson and Williams, 2018), suberin-like and lignin-like substances exhibit blue autofluorescence when excited by UV-A light (360 nm), and lignin-like substances exhibit green autofluorescence when excited by blue light (480 nm). At 360 nm and 480 nm, excitation revealed strong blue and green fluorescent signals on the water-facing sides of fronds (**Figures 6A-D**). Subsequently, a phloroglucinol staining test was performed, wherein the binding of phloroglucinol to polyphenol domains in lignin and suberin leads to autofluorescence quenching (Biggs, 1984). **Figure 6H** shows that treatment with phloroglucinol results in nearly complete absence of fluorescent signals compared to a non-treated slice (**Figure 6G**), supporting the presence of the lignin- and/or suberin-like polyaromatic domains at the water-facing surface of duckweed fronds. **Figures 6E,F**

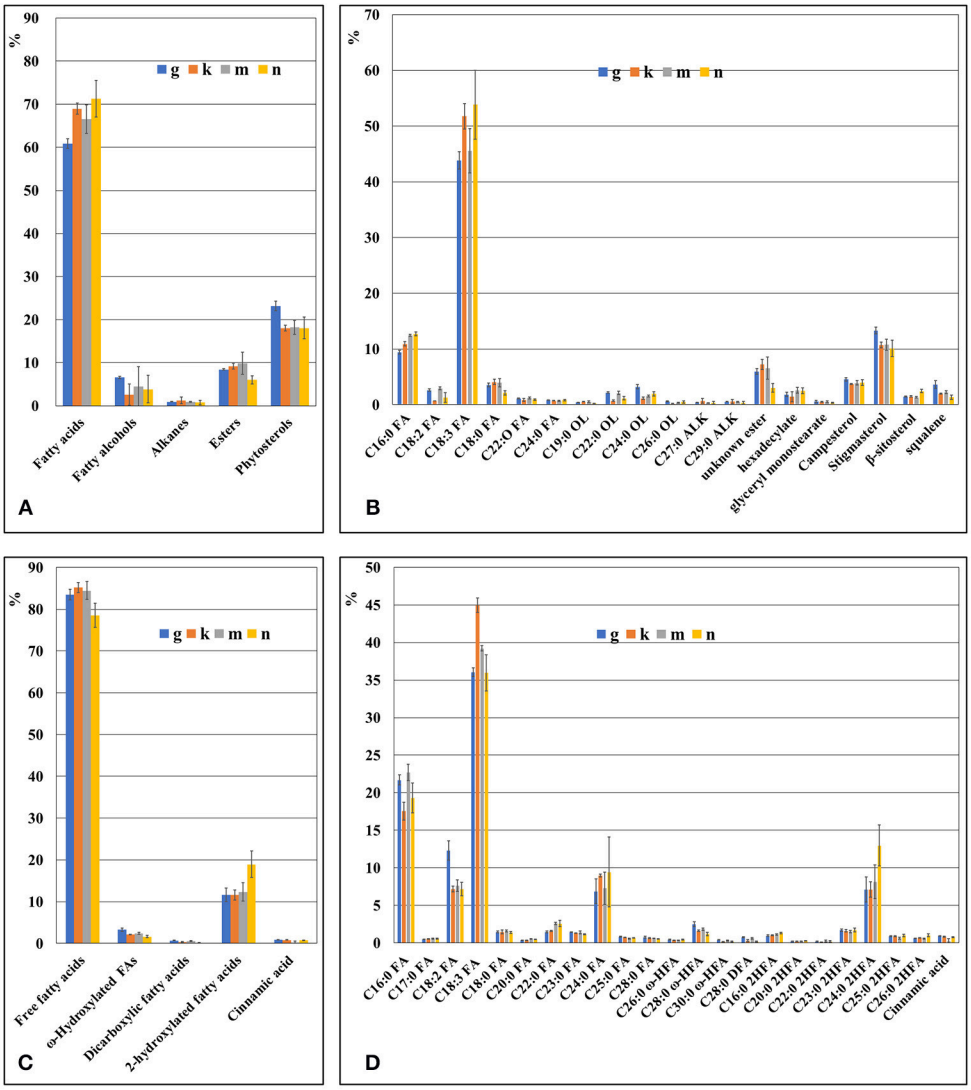

FIGURE 4 | The chemical composition of duckweed fronds cuticle as revealed by GS-MS and GS-FID analysis. **(A)** Components percentage in wax fraction of the duckweed fronds surface. **(B)** Percentage of individual components in the wax fraction of duckweed fronds surface. **(C)** Components percentage in the cutin fraction of duckweed fronds. **(D)** Percentage of individual monomer components in the cutin fraction of duckweed fronds surface. C16:0 FA, hexadecanoic acid; C18:2 FA, (9Z,12Z)-octadeca-9,12-dienoic acid; C18:3 FA, octadecatrienoic acid; C18:0 FA, octadecanoic acid; C20:0 FA, icosanoic acid; C22:0 FA, docosanoic acid; C23:0 FA, tricosanoic acid; C24:0 FA, tetracosanoic acid; C25:0 FA, pentacosanoic acid; C28:0 FA, octacosanoic acid; C19:0 OL, nonadecan-1-ol; C22:0 OL, docosan-1-ol; C24:0 OL, tetracosan-1-ol; C26:0 OL, hexacosan-1-ol; C27:0 ALK, heptacosane; C29:0 ALK, nonacosane; C26:0 ω HFA, ω-hydroxy hexacosanoic acid; C28:0 ω-HFA, ω-hydroxy octacosanoic acid; C30:0 ω-HFA, ω-hydroxy triacontanoic acid; C28:0 DFA, octacosanedioic acid; C16:0 2HFA, dihydroxy hexadecanoic acid; C20:0 2HFA, dihydroxy icosanoic acid; C22:0 2HFA, dihydroxy docosanoic acid; C23:0 2HFA, dihydroxy tricosanoic acid; C24:0 2HFA, dihydroxy tetracosanoic acid; C25:0 2HFA, dihydroxy pentacosanoic acid; C26:0 2HFA, dihydroxy hexacosanoic acid.

show autofluorescence of the water-facing surfaces following NaOH treatment, which removes cell-wall-bound phenolic acids (Ride and Pearce, 1979), which can be another source of autofluorescence (Biggs, 1984). Almost no difference was found between the blue and green fluorescent signals of treated (**Figures 6E,F**) and untreated samples (**Figures 6C,D**), further suggesting that the major fluorescent signals did not originate from cell-wall-bound phenolic acids, but rather lignin- and/or suberin-like compounds.

DISCUSSION

The cuticle, a continuous protective skin that covers all aerial surfaces of plants and serves as the interface between plant tissues and the environment, has been investigated in many aspects for a number of plant species and organs and at various developmental stages (Kunst and Samuels, 2009; De Luca and Valacchi, 2010; Ingram and Nawrath, 2017). Cutin and cuticular waxes composed of various long-chain (C20–C40)

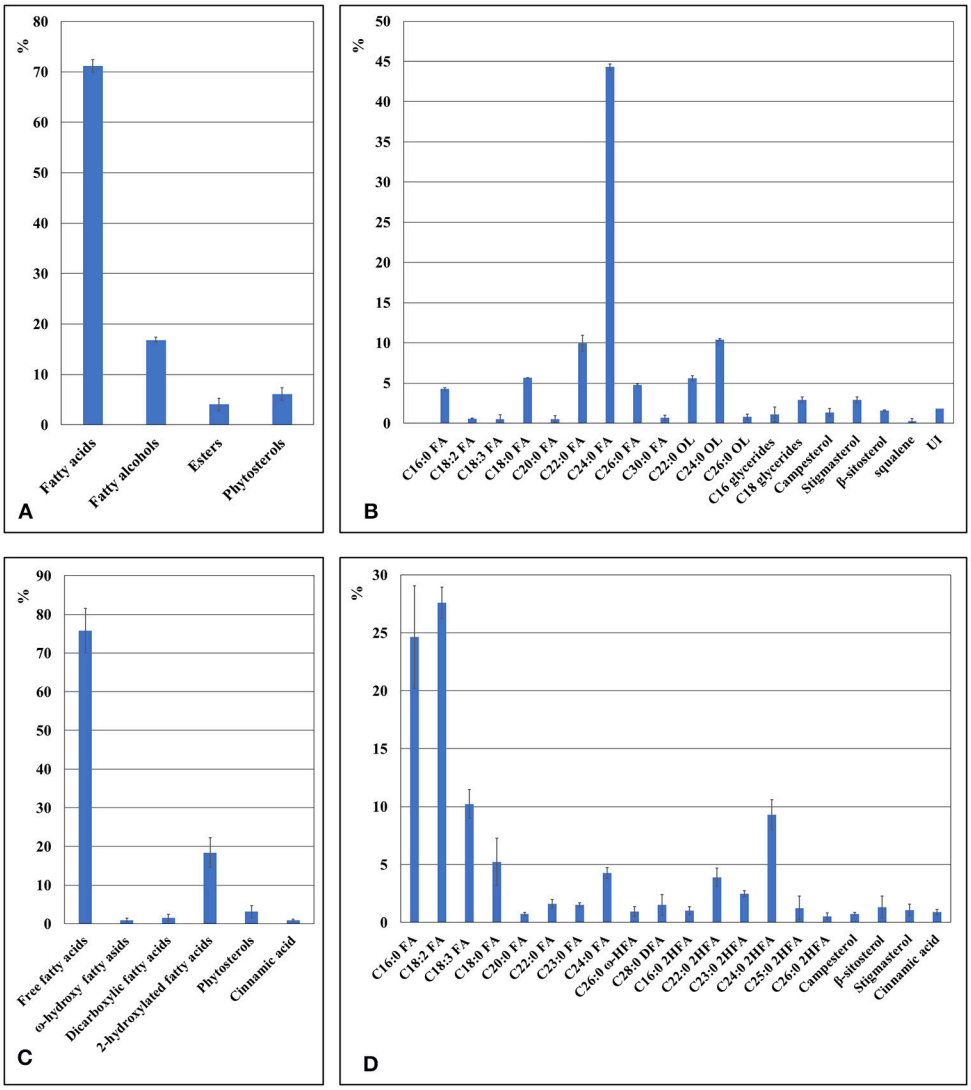

FIGURE 5 | The chemical composition of cuticle in duckweed turions cuticle as revealed by GS-MS and GS-FID analysis. **(A)** Components percentage in wax fraction of the duckweed turions surface. **(B)** Percentage of individual components in the wax fraction of duckweed turions surface. **(C)** Components percentage in the cutin fraction of duckweed turions. **(D)** Percentage of individual monomer components in the cutin fraction of duckweed turions surface. C16:0 FA, hexadecanoic acid; C18:2 FA, (9Z,12Z)-octadeca-9,12-dienoic acid; C18:3 FA, octadecatrienoic acid; C18:0 FA, octadecanoic acid; C20:0 FA, icosanoic acid; C22:0 FA, docosanoic acid; C23:0 FA, tricosanoic acid; C24:0 FA, tetracosanoic acid; C26:0 FA, hexacosanoic acid; C30:0 FA, triacontanoic acid; C19:0 OL, nonadecan-1-ol; C22:0 OL, docosan-1-ol; C24:0 OL, tetracosan-1-ol; C26:0 OL, hexacosan-1-ol; C26:0 ω-HFA, ω-hydroxy hexacosanoic acid; C28:0 ω-HFA, ω-hydroxy octacosanoic acid; C30:0 ω-HFA, ω-hydroxy triacontanoic acid; C28:0 DFA, octacosanedioic acid; C16:0 2HFA, dihydroxy hexadecanoic acid; C22:0 2HFA, dihydroxy docosanoic acid; C23:0 2HFA, dihydroxy tricosanoic acid; C24:0 2HFA, dihydroxy tetracosanoic acid; C25:0 2HFA, dihydroxy pentacosanoic acid; C26:0 2HFA, dihydroxy hexacosanoic acid; UI(VE)-UI, unidentified substances.

fatty acid derivatives (primary and secondary alcohols, alkanes, aldehydes, and esters) are recognized as the primary cuticle components (Domínguez et al., 2011). Within those components, a considerable variability in biochemical composition is found based on plant species (Jetter et al., 2007). This variability is primarily defined by the variant combination of basic aliphatic components and the incorporation of additional compounds such as terpenoids, flavonoids, and phenolic lipids (Hunt and Baker, 1980). Moreover, cuticles of particular plant groups contain taxon-specific components. For example, grasses are characterized by significant amounts of ß-diketones and related compounds (von Wettstein-Knowles, 2012; Bi et al., 2017). Current knowledge of cuticle structure and function originates almost exclusively from studies of terrestrial plants, for which the primary role of the cuticle is protection against water loss.

However, a substantial portion of species, the aquatic plants (or hydrophytes), inhabit water-based environments such as ponds, lakes, and other small reservoirs. Because of their unlimited water supply, protection against water loss

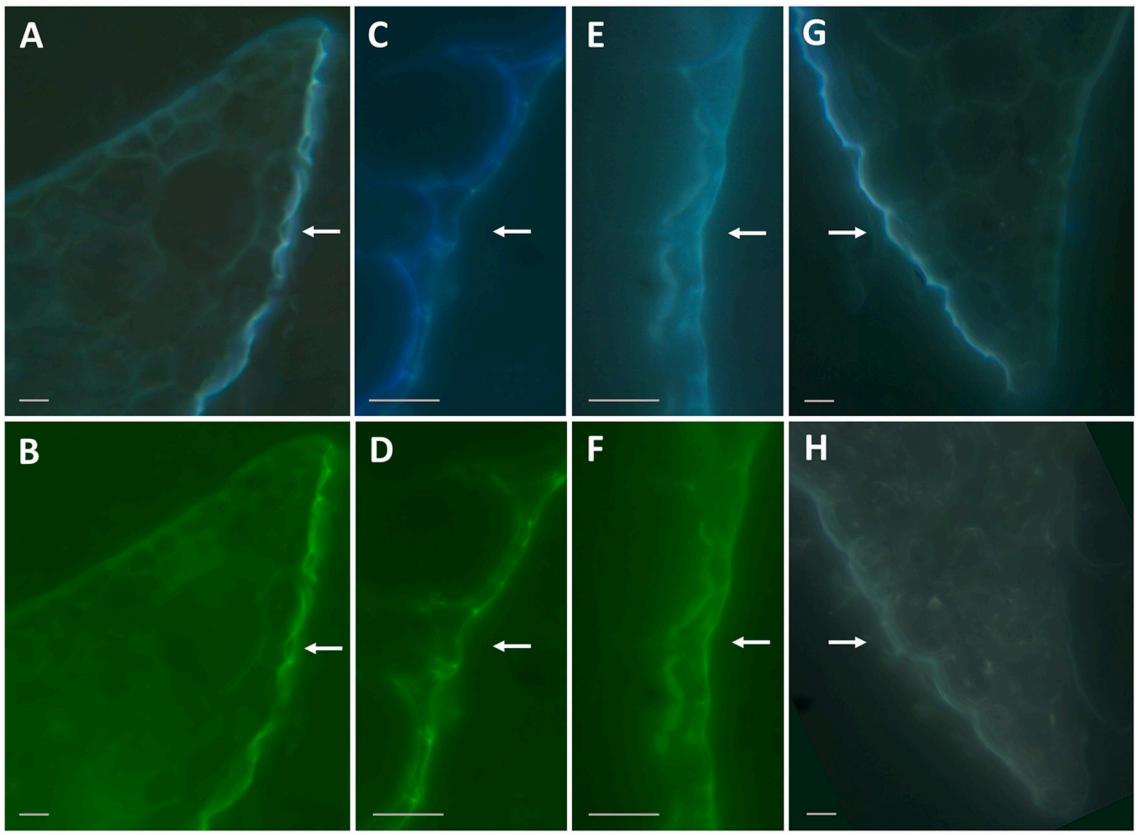

FIGURE 6 | Autofluorescence of duckweed frond sections under 360 nm and 480 nm excitation. **(A,B)**: Representative images of cross-section of the frond edge with autofluorescence excited by 360 nm **(A)** and 480 nm **(B)** wavelength light. **(C,D)**: Same as **(A)** and **(B)** under higher magnification. **(E,F)**: Representative images of cross-sections of the fronds treated with 0.5 M NaOH and excited by 360 nm **(E)** and 480 nm **(F)** wavelength light. **(G,H)**: Representative images of cross-sections of the fronds excited by 360 nm wavelength light without any treatment **(G)** and following a treatment with phloroglucinol-HCl **(H)**. Bars correspond to 10 μm on the images. Arrows point to the water-facing side of duckweed fronds.

is not as relevant for this group of flora. However, because aquatic floating-leaf plants adapted to growth on the water surface in a fixed position, they have no mechanism to protectively adjust their leaves and other organs relative to sunlight. Therefore, the surfaces of hydrophytes such as duckweed should function as effective protectors against potentially damaging UV irradiation. This is especially true in tropical and subtropical regions such as south China, where increased amounts of solar radiation are found.

In this study, we investigated the properties of the duckweed cuticle in four ecotypes grown around Hongze Lake in Jiangsu province, China by compositional analysis using GC-MS/GC-FID and cryo-SEM. Samples isolated from four locations were genotyped using two chloroplast markers and nuclear ribosomal DNA markers. The chloroplast DNA markers identified the samples as the greater duckweed, *Spirodela polyrhiza*, a dominant duckweed species in southern and middle China (Tang et al., 2015). The chloroplast ATP barcode showed some variability between samples, characteristic for the sequence variation between ecotypes of the same species (Supplement Figure 1). Similar variations were detected in nuclear 5S rDNA spacer sequences (**Figure 1B**). Correspondingly, compositional analysis of the wax and cutin cuticle components showed curtain levels of variations between ecotypes (**Figure 4**). Statistical analysis using the GLM multivariate test confirms that the majority of the wax and cutin components found in the four duckweed isolates can be quantitatively grouped within unique homogeneous domains (Supplement Tables 1, 2).

Chemical analyses of chloroform-extracted cuticle surface waxes revealed fatty acids, primary alcohols, esters, and alkanes (**Figure 4A**), typical constituents found in the cuticles of terrestrial plants, as the major cuticle components in *S. polyrhiza*. However, based on two extraction times, results show that the proportions of these components depend on the contact time between the fronds and the chloroform extractant. At the longer extraction time, the most prevalent component was the various types of free fatty acids (65%), followed by phytosterols (20%) dominated by C18:3 (linolenic acid) and C16:0 (palmitic acid; **Figures 4A, B**). At the shorter extraction time, phytosterols were the primary components in the fronds (about 60%), and the fatty acids followed in abundance (18.8%), dominated by saturated C16:0, C18:0, and C24:0. Such a

difference in the proportion of fatty acids most probably reflects the fragile nature of *S. polyrhiza* fronds, compared to other plants, such as wheat (Bi et al., 2016), and the high proportion of fatty acids in the samples extracted for longer time most probably resulted from lipids leaking from internal tissues. Therefore, additional tests using the careful enzyme-assisted separation of cuticle (Guzman et al., 2014) are needed to gain a more accurate estimation of cuticle components.

Phytosterols have been identified in surface extracts of many plants (Jetter et al., 2007), however the high percentage (up to 60%) of phytosterols represented by campesterol, stigmasterol, sitosterol, and their common precursor squalene in the wax fraction of duckweed is rather surprising. As recently reported by Appenroth et al. (2017), phytosterols represent a significant part, 5%, of the total lipids in *Wolffia*, another duckweed representative. This is one of the highest fractions found in vegetative oils (Phillips et al., 2002), indicating these compounds potentially have important roles in the Lemnaceae family. Phytosterols, isoprenoid-derived molecules linked with fatty acid carbon chains of varying length, have been associated with the response to various abiotic stresses and non-host resistance to bacterial pathogens. For example, squalene is quite abundant in human skin where, together with free fatty acids, it represents the first line of defense against solar radiation by directly absorbing UV radiation, and it acts as an efficient scavenger for reactive molecular species generated by that radiation (De Luca and Valacchi, 2010).

In the turions, fully-saturated C24:0 was revealed as the most dominant fatty acid in the cuticle (**Figure 5**). Polyunsaturated linolenic fatty acid is a rather unusual component in plant cuticle. This class of fatty acids is more characteristically found among storage lipids and/or for membrane lipids. Evidence shows there is polyunsaturated C18:3 fatty acids present in plant surface structures: Hernández-Pinzón et al. (1999) indicated the abundance of these molecules in the polar soluble lipid fraction of the pollen coat (68.2%), and it was shown that this compound was relocated to the pollen surface during tapetum programmed cell death from the elaioplasts (Wu et al., 1997).

Among the cutin monomers in the duckweed fronds, a substantial amount was C18:3 (α-linolenic acid), but almost 15% was fully saturated longer-chain (24C) fatty acids and hydroxyl-fatty acids (**Figures 4C,D**). In the turion cutin, the most abundant fatty acids were C18:2 and fully-saturated C16:0. Though polyunsaturated fatty acids are not often found among cutin monomers, small amounts were detected in *Arabidopsis thaliana* (Franke et al., 2005; Fich et al., 2016). The major part of the fatty acids among the suberin monomers coincides with those in the cutin monomers (Graça, 2015). Cinnamic acid is an usual aromatic monomer of the polyaromatic domain in suberin and lignin (Bernards et al., 1995). Its detection in duckweed cutin could indicate suberized layers in addition to the cuticle.

Lignin-like and suberin-like substances are among of most native fluorophores in plant tissues (Donaldson and Williams, 2018). In duckweed fronds, we found that autofluorescence indicated the presence of polyaromatic structures such as suberin or lignin in the epidermal layer of the water-facing side (**Figure 6**). These types of substances are common in plant roots (Wilson and Peterson, 1983). The water-facing side of the duckweed fronds performs root-specific functions; therefore, it is not surprising that the chemical composition of this frond surface shows similarity to that found at the root surface.

Using cryo-SEM, we observed wax crystals on the regular backbone film structure of the *Spirodela* cuticle surface (**Figure 2**). The cuticle film on the adaxial side of the frond is most probably composed of fatty acids, the dominant component of duckweed waxes. We speculate that the wax crystals are composed of fatty alcohols, giving their ability to form surface crystals (Koch et al., 2006). After all, fatty alcohols were the second-most abundant chemical group in the wax, following fatty acids. The barely-distinguished nanoridge net on the native adaxial side of the fronds became profoundly visible on SEM images of samples treated with chloroform to remove the wax. On the contrary, at the abaxial side of the fronds emerged in water, SEM revealed a well-structured net-like structure covering the epidermal cells (**Figure 2**).

Similarly, an even more profound structure was detected on the abaxial side of *Spirodela* turions (**Figure 3**). To the best of our knowledge, for neither fronds nor turions can this net-like structure be assigned to a previously classified type of epicuticle "superimposed" wax structure. Following treatment with chloroform, this net remains intact. The chemical nature of the net components remains unknown and the subject of future investigations.

In summary, this study elucidated the structural and biochemical compositions of the cuticle in duckweed, *S. polyrhiza*. This is the first data published of this type, to the best of our knowledge, representing aquatic plants.

Microscopic investigations of the duckweed surfaces (cryo-SEM and fluorescence microscopy) uncovered significant differences in structures between the abaxial (water-facing) and adaxial (air-facing) sides of the plant's fronds. While the adaxial side, with multiple stomata complexes, is a smooth continuous sheet with randomly-distributed wax crystals, a rather typical structure for terrestrial species; the abaxial side is built from large oval cells covered with a distinct net-like structure resistant to chloroform and possessing autofluorescence characteristics of suberin and/or lignin.

Analysis using GC-MS of chloroform-extracted cuticle revealed a unique biochemical composition of cuticular waxes primarily identified via GC-MS/GC-FID as fatty acids and phytosterols (up to 60%). This high phytosterol content raises special interest because of their functions in the absorption of UV irradiation and scavenging of UV-generated radicals (De Luca and Valacchi, 2010) in addition to their potential in lowering human serum cholesterol (Kritchevsky and Chen, 2005).

Because the genome sequence of *S. polyrhiza* (Wang et al., 2014; Michael et al., 2017) is available, duckweed could be used as a model for studying fundamental processes related to plant protection against UV radiation and other environmental

stresses. It could also be a promising platform for producing valuable products such as phytosterols.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NB and JS. Performed the experiments: JS, AP, JL, GQ, QL, LS, GC, OK and YZ. Analyzed the data: JS, AP, and NB. Contributed reagents, materials, and analysis tools: JS, YZ and NB. Wrote the paper: NB, AP and JS.

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Metabolic Patterns in *Spirodela polyrhiza* Revealed by ^{15}N Stable Isotope Labeling of Amino Acids in Photoautotrophic, Heterotrophic, and Mixotrophic Growth Conditions

Erin M. Evans[1,2], *Dana M. Freund*[1,2], *Veronica M. Sondervan*[1], *Jerry D. Cohen*[1,2] and *Adrian D. Hegeman*[1,2,3]*

[1] Department of Horticultural Science, University of Minnesota, Twin Cities, Saint Paul, MN, United States, [2] Plant and Microbial Genomics Institute, University of Minnesota, Twin Cities, Saint Paul, MN, United States, [3] Department of Plant and Microbial Biology, University of Minnesota, Twin Cities, Saint Paul, MN, United States

In this study we describe a [^{15}N] stable isotopic labeling study of amino acids in *Spirodela polyrhiza* (common duckweed) grown under three different light and carbon input conditions which represent unique potential metabolic modes. Plants were grown with a light cycle, either with supplemental sucrose (mixotrophic) or without supplemental sucrose (photoautotrophic) and in the dark with supplemental sucrose (heterotrophic). Labeling patterns, pool sizes (both metabolically active and inactive), and kinetics/turnover rates were estimated for 17 of the proteinogenic amino acids. Estimation of these parameters followed several overall trends. First, most amino acids showed plateaus in labeling patterns of <100% [^{15}N]-labeling, indicating the possibility of a large proportion of amino acids residing in metabolically inactive metabolite pools. Second, total pool sizes appear largest in the dark (heterotrophic) condition, whereas active pool sizes appeared to be largest in the light with sucrose (mixotrophic) growth condition. In contrast turnover measurements based on pool size were highest overall in the light with sucrose experiment, with the exception of leucine/isoleucine, lysine, and arginine, which all showed higher turnover in the dark. K-means clustering analysis also revealed more rapid turnover in the light treatments with many amino acids clustering in lower-turnover groups. Emerging insights from other research were also supported, such as the prevalence of alternate pathways for serine metabolism in non-photosynthetic cells. These data provide extensive novel information on amino acid pool size and kinetics in *S. polyrhiza* and can serve as groundwork for future metabolic studies.

Keywords: stable isotope, nitrogen, *Spirodela polyrhiza*, duckweed, autotrophic, heterotrophic, mixotrophic, amino acids

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United Kingdom

***Correspondence:**
Adrian D. Hegeman
hegem007@umn.edu

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INTRODUCTION

Primary metabolism in plants is responsible for providing the cell with fixed carbon, energy, reduced cofactors for cellular reactions, and all the building blocks for secondary metabolism and production of biomass. This portion of metabolism, including glycolysis, the citric acid cycle, the pentose phosphate pathway, amino acid metabolism, and fatty acid synthesis are

relatively conserved across species (Peregrín-Alvarez et al., 2009). Amino acid metabolism is a unique portion of metabolism as it bridges carbon and nitrogen metabolism and amino acids are important intermediates in many metabolic processes. Alanine interacts with glycolysis and the citric acid cycle via pyruvate (Schulze-Siebert et al., 1984). Asparagine and its derivatives link through oxaloacetate (Lea and Fowden, 1975). Serine connects through photorespiration and feeds into one-carbon/folate metabolism (Ros et al., 2014). Arginine plays a key role in nitrogen storage and in the urea cycle (Witte, 2011). The aromatic amino acids feed into portions of secondary metabolism, namely phenylpropanoid metabolism, and lignin biosynthesis and tryptophan, specifically, provides a substrate for auxin biosynthesis (Maeda and Dudareva, 2012; **Figure 1**).

One of the most fascinating qualities of central metabolism in the context of plants is that they have the ability to run primary metabolism as both autotrophs and heterotrophs. The most basic examples of this are the differences between shoot (metabolic source) and root (metabolic sink) tissue (Ho, 1988; Sonnewald and Fernie, 2018) and the shifts in metabolism that occur with diurnal cycles when photosynthesis cannot occur (Geiger and Servaites, 1994). These changes have the potential to reveal how plants manage their resources such as fixed carbon from CO_2 in the air, and nitrogen, minerals, and water from the soil. Understanding these plant resource allocation practices can have a potentially large impact on food and plant production (Sonnewald and Fernie, 2018) as the end products of central metabolism are the main components of biomass: starch, lipids/fats, and proteins are also the main nutrients people seek in food. The ability to manipulate these core metabolites/nutrients may carry more weight than before as consumer demands and federal regulations of food products are beginning to shift toward healthier macronutrient profiles including improved lipid profiles (Unnevehr and Jagmanaite, 2008).

Light and dark effects on amino acid pools have been well established for a number of years due to many studies carried out in the 1960s through the 1980s (Singh, 1998). These studies have assessed amino acid pool sizes in the context of whole plants over the day-night cycle. Each of these studies has shown strong variation in amino acid pool sizes between day and night. The general trend, however, showed that free amino acid pool sizes tended to be lower during the dark night hours (Noguchi and Tamaki, 1962; Bauer et al., 1977). This contrasts with studies of chlorophyll synthesis mutants in barley and corn which exhibit accumulation of almost all of the free amino acids (Maclachlan and Zalik, 1963; Shortess and Amby, 1979). Additional studies in *Zostera marina* root tissue indicated key amino acids, such as glutamate/glutamine and alanine having lower pool sizes in the dark (Pregnall et al., 1987). More recent studies on the transcriptional regulation of key enzymes involved in amino acid biosynthesis support these earlier light/dark amino acid studies (reviewed in Coruzzi, 2003).

Metabolic models are curated maps representing how the chemical reactions of metabolism are connected. Resent advances in using annotated genomes to build these networks in a number of plant species including *Arabidopsis thaliana* (Poolman et al., 2009; de Oliveira Dal'Molin et al., 2010a; Arnold and Nikoloski, 2014), corn (de Oliveira Dal'Molin et al., 2010b), and developing *Brassica napus* embryos (Schwender et al., 2003) have ignited an expansion in the field of metabolic flux and network analysis which seeks to estimate the quantitative flow of matter through metabolic reactions. Many of these advances have been in the realm of stoichiometric methods, such as flux balance analysis and they have been possible because of the emerging high-quality metabolic models (Sweetlove and Ratcliffe, 2011). These methods have been used to elucidate costs associated with amino acid biosynthesis under different nitrogen and metabolic growth conditions (Arnold et al., 2015), resource allocation during seed filling (Schwender et al., 2006), and overall metabolic patterns in central metabolism (Szecowka et al., 2013). However, these studies have had the advantage of having been conducted with species that have well-developed genomes as well as genome-scale metabolic models. In contrast, there are many organisms that do not have these well-developed system models.

The species of interest for this study is the common duckweed (*Spirodela polyrhiza*), a small aquatic angiosperm and a member of the family *Lemnaceae* which contains five genera: *Landolita, Lemna, Spirodela, Wolffia,* and *Wolffiella*. The *Lemnaceae* are monocotyledonous plants that reproduce primarily through asexual budding (Appendroth et al., 2013). They are also among the fastest growing angiosperms and are capable of doubling biomass in 16–24 h depending on the conditions (Peng et al., 2007). Additionally, they are able to accumulate high starch content of up to 75% of the dry weight (Reid and Bieleski, 1970; Xu et al., 2012b) and high-protein content. This ability to accumulate a large percentage of biomass as starch has led to some commercial interest in duckweed species as biofuel feedstock and it has been shown that it is possible to increase this starch content under nutrient stresses (Reid and Bieleski, 1970; Cui and Cheng, 2015) and also under certain light regimens (Yin et al., 2015). Duckweed species also grow readily on wastewater. A particular example of growth of three isolates on swine lagoon water (Bergmann et al., 2000; Cheng et al., 2002; Xu et al., 2011) highlights the possibility of mixing duckweed production into existing agricultural pipelines. Additional commercial interests for duckweeds include bioremediation (Oron, 1994) and recombinant protein production (Xu et al., 2012a). Because of the utility of duckweed for many uses it is going to be increasingly important to have an understanding of the full extent of metabolism, including metabolic rates/fluxes, static pool sizes, and information on how these respond to different growth and metabolic conditions such as differing light treatments.

In this study, we present a metabolic [^{15}N]-labeling study of *S. polyrhiza* grown under three metabolic growth conditions: growth in light cycle on medium with supplemental sucrose (mixotrophic condition), growth in light cycle on medium without supplemental sucrose (photoautotrophic condition), growth in dark on medium with supplemental sucrose (heterotrophic condition). This growth set-up allows us to accomplish several objectives. First, our aim was to profile

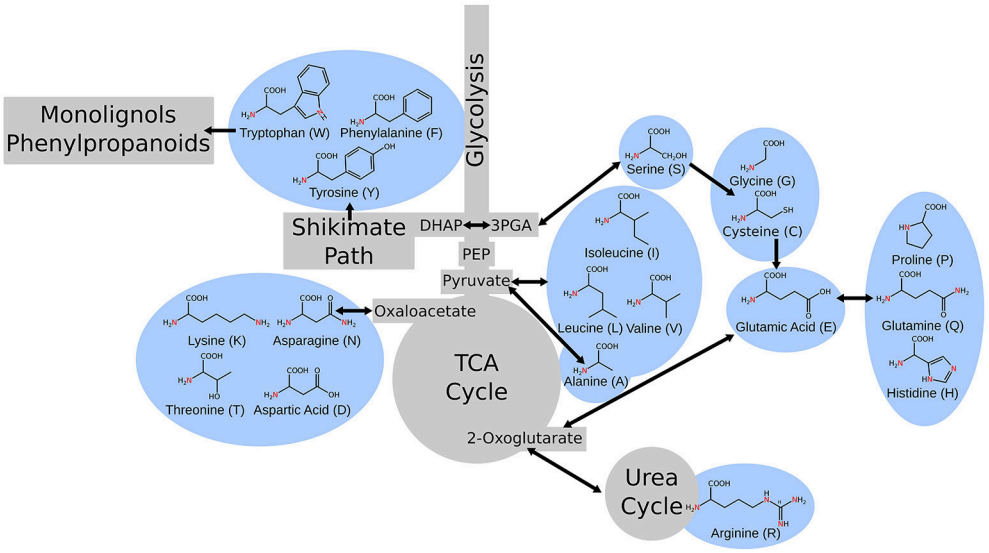

FIGURE 1 | A simple schematic of central metabolism in the context of carbon flow showing the points where the metabolism of various amino acids intersect.

nitrogen metabolism and flow through amino acids and [^{15}N]-labeling patterns over time to determine differences in the metabolic patterns between growth conditions. The second, aim was to estimate pool size and turnover information for amino acids in each condition and identify changes between growth conditions. The third aim was to determine if broad patterns in nitrogen flow exist between growth treatments that can be captured through clustering analysis. *S. polyrhiza* and other duckweeds have a history of use in stable isotopic labeling studies (Rhodes et al., 1981; Baldi et al., 1991; Rapparini et al., 1999) and can readily incorporate label from liquid growth medium. The [^{15}N]-labeling study of *S. polyrhiza* facilitated the ability to measure amino acid pool sizes, turnover numbers, and adjusted pool sizes and to compare between experimental conditions. Here we lay the groundwork for further in-depth metabolic studies as well as reveal insights into central metabolism in duckweed.

MATERIALS AND METHODS

S. polyrhiza Material and Long-Term Culture Maintenance

All duckweed cultures used in this work were clonally propagated *Spirodela polyrhiza* [ID number 7498 from the Rutgers Duckweed Stock Cooperative (http://www.ruduckweed.org/)] maintained on Schenk and Hildebrandt (SH) medium prepared from SH Basal Salt Mixture (Sigma-Aldrich St. Louis, MO) with 10% w/v sucrose and 1% agar with the pH adjusted to 5.8 using potassium hydroxide. Cultures were kept at 15°C, with a 15 h light cycle under white light from an LED array with light intensity of 17 μmol/m^2•s.

Experimental Growth Conditions

For experiments conducted in the light, actively growing duckweed cultures were maintained on sterile medium prepared from SH Basal Salt Mixture (Sigma-Aldrich St. Louis, MO) with the pH adjusted to 5.8 using potassium hydroxide, either with or without 10% w/v sucrose depending on experimental conditions. Cultures were maintained in a controlled growth environment for 4–6 weeks, under a 16/8 day/night cycle of cool white florescent light at an intensity of 67 μmol/m^2•s at 22°C. Light was measured approximately at the level of the cultures. Cultures were determined to be saturated when the growth was covering the entire surface with little to no medium visible when viewed from above. Upon saturation the culture was transferred to labeled medium as described below.

For experiments conducted in the dark, actively growing duckweed cultures were maintained on sterile SH medium prepared from SH Basal Salt Mixture at pH 5.8 (Sigma-Aldrich St. Louis, MO) supplemented with 3.01 × 10^{-3} mM kinetin as used for *Lemna gibba* (Slovin and Tobin, 1982) in a dark box at room temperature under 2 min of red light every 8 h (10 μmol/m^2•s) at room temperature. Cultures were only removed from the dark box under green light. When the cultures reached saturated growth, the culture was transferred to labeled medium for sampling as described below.

[^{15}N]-Labeling Experiment Medium Preparation

[^{15}N]-labeled modified SH with a) no added sucrose; b) 10% w/v sucrose; c) 10% w/v sucrose and 3.01 × 10^{-3} mM kinetin, was prepared according to the specifications in Table S1. Nitrogen was provided as 80.7% K[^{15}N]O_3 and 19.3% [^{15}N]H_4 [^{15}N]O_3. The pH of the solution was adjusted to 5.8 with potassium hydroxide and autoclaved. The necessary iron components were added by

syringe filter sterilization after the medium cooled to minimize precipitation of medium components.

Duckweed Transfer and Sampling Procedure

Experiments With a Light Cycle

Duckweed from actively growing cultures ± sucrose were decanted from the culture under aseptic conditions and washed with sterile distilled deionized water. Duckweed fronds were then sorted into five groups and transferred into either five 2 L Pyrex bottles with 300 mL of medium, or five 500 mL Pyrex bottles with 100 mL of medium. Once duckweeds were transferred, initial samples were taken from the extra plant material not needed for the labeling and transferred to pre-weighed 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Samples were taken at the following time points: 0, 1, 2, 4, 8, 16, 32, 64, and 128 h. Additional samples were also taken at 15 and 30 min timepoints from cultures grown without added sucrose. Each sample consisted of at least three duckweed fronds. Snap frozen samples were stored at −80°C until extraction and LC-MS analysis. Two experiments were carried out for the light without sucrose conditions, which were started at different times in the day. One of these, started later in the day, was used for pool size calculation, and the other, started near the beginning of the light cycle in line with the other light experiment, was used to estimate label enrichment and estimate the kinetic model. This growth contained four replicates rather than five.

Experiments in the Dark

The transfer and sampling procedures took place the same as for light cycle experiments with the following exceptions. Sampling was carried out in the dark under green light. Samples were taken at the following time points: 0, 15, and 30 min, and 1, 2, 4, 8, 16, 32, 64, and 128 h.

Microscopy

Duckweed plants were grown in either light or dark conditions for at least 2 weeks. When cultures reached saturation individual duckweed fronds were removed and thin layers of leaf tissue were scraped off with a clean razor and put onto a microscope slide. Brightfield and fluorescence images were taken at 1,000x magnification with Leitz Laborlux D (Stuttgart, Germany) fluorescence microscope. Fluorescence images were acquired with an N2.1 filter to visualize chlorophyll [excitation wavelength range: 515–560 nm (green); emission wavelength: >590 nm (red)]. Image J was used to make montages, overlays, and to add scale bars (Schindelin et al., 2012, 2015).

Sample Extraction

Frozen sample weights were taken. Samples were kept on dry ice and up to 1.5 mL/mg sample fresh weight cold 70% isopropanol was added to each sample. Samples were then homogenized in a Geno/grinder tissue homogenizer at 1,500 rpm for 5 min. Sample homogenate was then centrifuged at room temperature for at least 3 min and supernatant was decanted into a fresh microcentrifuge tube and stored at −80°C until mass spectral analysis.

Dry Residue Analysis

100 or 200 μL of sample extract was added to a pre-weighed 1.5 mL microcentrifuge tube and the samples were then dried under vacuum and the weight of the residue was determined. Samples were reconstituted to a concentration of 2 mg sample extract/mL in 70% isopropanol and stored at −80°C until mass spectral analysis.

LC-MS Analysis

LC Sample Preparation

Extracted samples were removed from storage and centrifuged at room temperature for at least 2 min to settle particulate matter. Samples were then loaded into LC-MS autosampler vials. Fresh plant extracts were diluted 1:10 in 70% isopropanol. Dried extract samples were diluted to a concentration of 1 μg/μL in 70% isopropanol in the light experiment without supplemental sucrose and the dark experiment and to a concentration of 0.9 μg/μL in the light with sucrose experiment. A commercially available [^{15}N]/[^{13}C]-labeled cell free mixed amino acid standard (Sigma-Aldrich St. Louis, MO) containing all proteinogenic amino acids was added to dry residue samples for a final concentration of 0.02 mM. All measurements were carried out on five biological replicates except in the light with sucrose experiment, which had only three replicates.

LC-MS Analysis

For each sample, 1 μL was injected onto a SeQuant ZIC-cHILIC column, 3 μm particle size, 100 × 2.1 mm using an Ultimate 3000 UHPLC system coupled to a Q-Exactive quadrupole-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization source. A 20 min gradient at a flow rate of 0.4 mL/min with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) with the following gradient: −2–0 min: 90% B, 0 min: 85% B, 18 min: 40% B, 18–20 min: 40% B. MS analysis used the following settings: full scan mode in positive ionization with a scan range of 50–750 *m/z*, a resolution of 70,000, a target automatic gain control of 1×10^6, and a maximum fill time of 200 ms. Data were collected using Thermo *Xcalibur* software version 4.0. Amino acid identity was verified through comparison of retention times and accurate mass to a commercially available mixed amino acid standard. The amino acid isomers of leucine and isoleucine are quantified together because of the inability to separate these with the chromatography system employed.

Data Analysis

Exact Mass Calculation and Retention Time Determination

Exact masses for both labeled and unlabeled amino acids were calculated using the University of Wisconsin—Madison Biological Magnetic Resonance Data Bank exact mass calculator (http://www.bmrb.wisc.edu/metabolomics/mol_mass.php). Retention times and exact mass measurements of

all amino acids were confirmed using amino acid standard H (Sigma-Aldrich, St. Louis, MO).

MS File Conversion and Data Extraction

Data files were converted from *.RAW* files to *.mzXML* files using the "msconvert" function of *Proteowizard* (Kessner et al., 2008) prior to input into *R*. Data from each amino acid was extracted through use of a pair of scripts developed in the Hegeman lab currently available on GitHub (https://github.com/orgs/HegemanLab files "metabolite turnover" and "clustering"). Briefly, we utilized the *ProteinTurnover* (Fan et al., 2016) and the *XCMS R* packages (Smith et al., 2006; Tautenhahn et al., 2008; Benton et al., 2010) to extract amino acid extracted ion chromatograms (EICs) for each amino acid isotopomer. These data were then used to generate labeling patterns tracking the decay of the unlabeled isotopomer (M0).

Data Modeling

Labeling patterns for M0 output by the clustering script were then used to model the labeling pattern of each amino acid via the "nls" function in *R*. The broom package was used to clean the data for export. The model implemented was a variation of the model by Yuan et al. (2006) for modeling a first-order decay of the unlabeled (M0) isotopomer:

$$M_0 = (1 - c)\, e^{-kt} + c$$

Where t is the labeling time in hours, k is the turnover constant, and c is a constant that defines the plateau of the labeling curve. Models were generated using time points 0–16 h except for the following cases that used 0–32 h: lysine in the dark grown experiment and 0–64 h: tryptophan in the light grown experiment without supplemental sucrose and alanine, arginine, threonine, and tryptophan in the dark grown experiment. Histidine was omitted from all modeling and valine was omitted from the light without sucrose and dark with sucrose experiments due to low data quality. All models were estimated using five biological replicates except in the light without sucrose experiment, which had only three replicates.

Clustering

K-means clustering was performed using *R*, time points between 0 and 32 h with k selected to maximize the between-group variation and to minimize the within group variation. The Hartigan-Wong algorithm was used to form clusters based on Euclidean distance. Further clustering was carried out based on the pool sizes adjusted for turnover. The number of clusters used for these analyses were the same as used for the first analysis except in the case of the light with sucrose experiment where four clusters rather than 3 were used.

Estimation of Pool Size, Pool Size Corrected for Turnover, and Active Pool Size

Pool sizes in all experiments were estimated from the initial time point to provide insight into the amount of available amino acid in each condition. Pool sizes were estimated relative to the labeled internal standard added to dry residue samples. All numbers for pool size carry the units μmol/mg sample extract residue.

The k-values from each model was used to estimate a flux value, denoted as pool size adjusted for turnover, for each amino acid as follows:

$$f_{AminoAcid} = k_{AminoAcid} \times P_{AminoAcid}$$

Where $f_{AminoAcid}$ is the flux estimate through an amino acid, $k_{AminoAcid}$ is the turnover number modeled for an amino acid, and $P_{AminoAcid}$ is the initial pool size estimated for an amino acid. All flux dimension numbers or turnover corrected pool sizes carry units of μmol/mg sample extract residue/hour.

Pool sizes were corrected to approximate an "active pool" as observed in illuminated Arabidopsis rosettes (Szecowka et al., 2013) and maize (Arrivault et al., 2016). The c component in the models were taken as an approximation of the proportion of the pool not active in metabolism and the pool size estimates were corrected as follows:

$$P_{Active} = P_{Initial} - (P_{Initial} - c)$$

Where P_{Active} is the estimated corrected pool size, $P_{Initial}$ is the originally estimated pool size, and c is the modeled plateau for each amino acid.

Pairwise Statistical Comparisons

Estimates for pool size and turnover adjusted pool size were compared across the three pairs of experimental conditions: light with sucrose vs. light without sucrose, light with sucrose vs. dark with sucrose, and light without sucrose and dark with sucrose. Comparisons were made via two-tailed student's t-test assuming unequal variance except where a F-test indicated equal sample variances in which case an equal variance test was applied.

RESULTS

[15N]-Labeling of *S. polyrhiza* Amino Acids in Phototrophic, Heterotrophic, and Mixotrophic Growth Conditions

In order to confirm the physiological effects of the light conditions duckweed grown for at least 2 weeks in light or dark conditions were sectioned and imaged under both brightfield and red fluorescence (**Figure 2**). Light grown samples exhibited normal chlorophyll autofluorescence and as expected dark grown samples had no detectable chlorophyll autofluorescence. [15N]-labeling was conducted once the growth conditions were confirmed as phototrophic, heterotrophic, and mixotrophic based on chlorophyll content. Fifteen amino acids were identified by utilizing LC-MS and retention time windows; their isotopomer molecular ion masses are in Table S2.

[15N]-Label Incorporation Models for Individual Amino Acids

Models for the incorporation rate of [15N]-label into each individual amino acid were generated. **Figure 3** contains a visual representation of each model and the values for the parameters of the models are listed in **Table 1**. General labeling patterns can be seen for all amino acids as well as between each

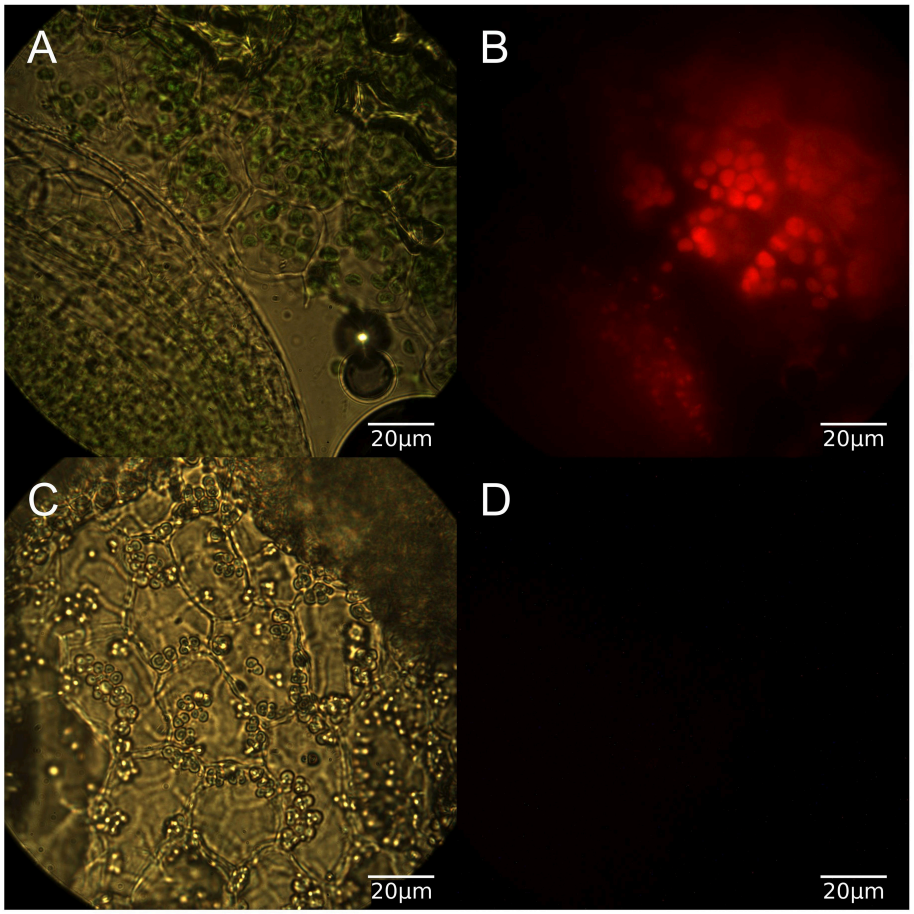

FIGURE 2 | (A) Brightfield microscopy of light grown duckweed at 100x, **(B)** Red fluorescence image of light grown duckweed, **(C)** Brightfield image of dark grown duckweed, **(D)** Red fluorescence image of dark grown duckweed.

experimental condition. Most amino acids exhibit a plateau or leveling-off phase around the 16 h time point with some amino acids, most notably glutamate, taking longer to reach that plateau. Dark grown samples generally exhibited slower turnover and higher plateau values with several amino acids, including alanine, asparagine, serine, and threonine not appearing to reach fully discernable plateau values within the timeframe measured Overall, the trends observed by modeling the rate of [^{15}N]-incorporation indicate there are both dramatic and subtle trends that are seen when duckweed is grown in phototrophic, heterotrophic, and mixotrophic conditions.

Estimation of Amino Acid Pool Size and Pool Size Adjusted for Turnover

Pool sizes in μmol/mg sample extract residue were estimated by comparison to an internal standard (Table S3). General patterns were observed through the experiments with the dark with sucrose condition exhibiting the largest pool sizes in almost every case where an amino acid was observed in the experiment (**Figure 4A**). However, the following amino acids are the exception: aspartic acid, glutamate, glycine, asparagine, and glutamine where the light with sucrose treatment exhibited larger pool sizes than other conditions. The light without sucrose treatment exhibited either the smallest or an intermediate pool size in every amino acid. Pool sizes can be explained to be either active or inactive. Active metabolite pools are those participating in metabolic reactions, while inactive pools are not and may be sequestered in a vacuole or otherwise unable to participate in metabolic reactions (Szecowka et al., 2013). Therefore, in addition to total pool sizes a total active pool size was estimated for each amino acid. For our purposes the active pool size was estimated by correcting the estimated pool size with the plateau value (c) of each model. Interestingly, following this correction the light with sucrose condition carried the largest pool size for all amino acids with the exception of lysine and arginine.

The turnover rates (estimated k-values) were multiplied by the initial pool size estimates for each amino acid to give a dimension of flux (μmol compound/unit time). This quantity is not a true flux value as it will encompass all enzymes and reactions involved in nitrogen transfer through each amino acid. The utility of the estimation is to allow direct comparison of

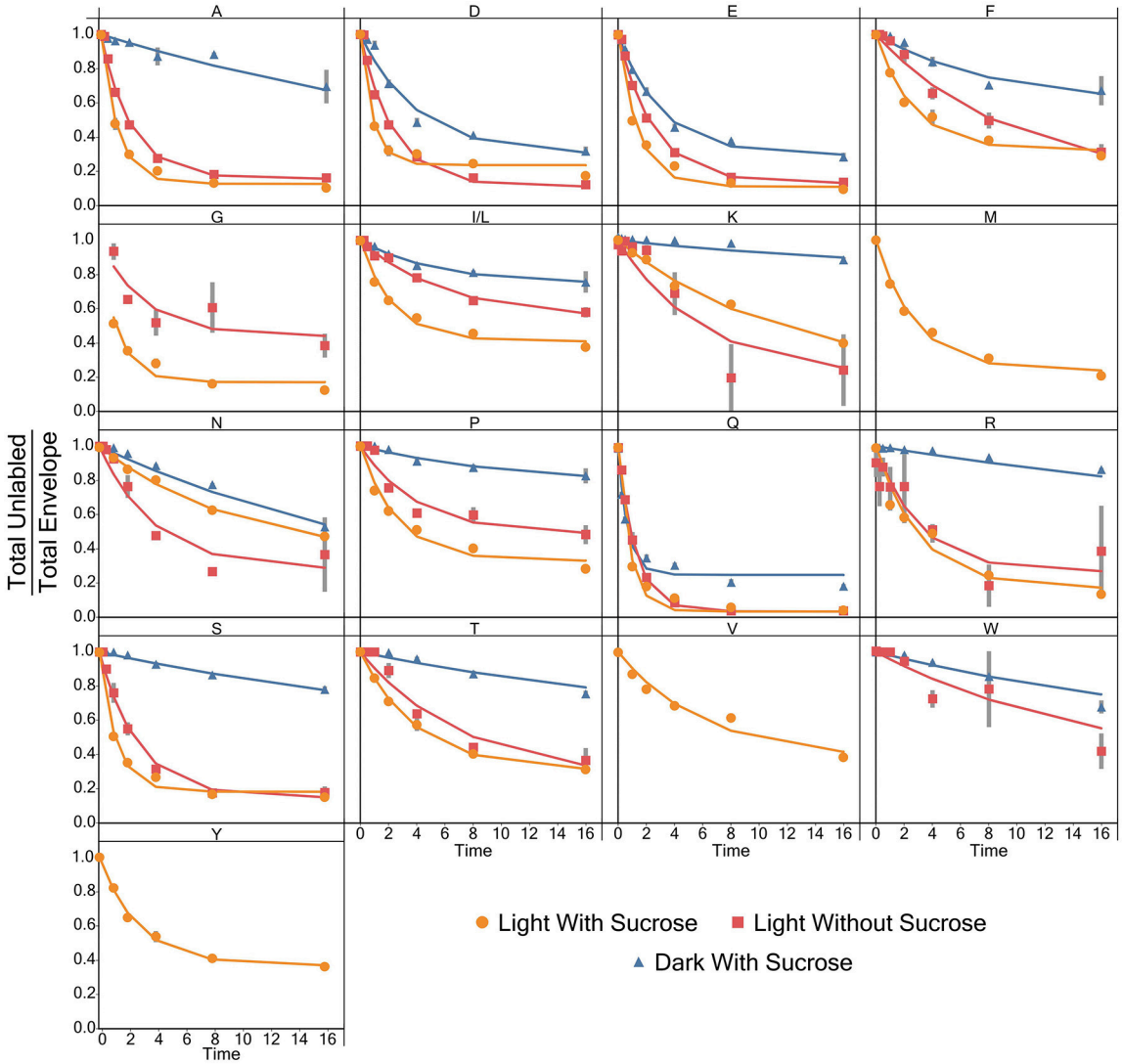

FIGURE 3 | A graph showing the average ratio of the unlabeled isotopomer to the total isotopic envelope for each amino acid in each experiment. The lines represent models generated in R. Error bars represent ± 1 standard error.

turnover rates between amino acids that may have dramatically different pool sizes. Turnover rates adjusted for pool size in this way in contrast to regular pool size estimates were higher in the light with sucrose experiments in almost all cases (Table S3 and **Figure 4B**). The exceptions were isoleucine/leucine, lysine, and arginine which both showed larger turnover values in the dark. Similar to the pool size estimation the light without sucrose treatment exhibited either the smallest or an intermediate pool size in every case. However, the light with sucrose and light without sucrose measurements for asparagine were nearly identical.

Turnover rates were also corrected using the active pool size and the results are summarized in Table S3 and **Figure 4B**. These results largely mirror the uncorrected total pool sizes with a few interesting differences. Lysine showed a compressing of the differences between estimates with the dark grown pool now slightly larger than the light with sucrose pool. In several cases the differences observed in pool sizes between the dark grown and light were reduced to the point it became visually indistinguishable. Most notably in aspartic acid, glutamate, proline, and asparagine. In addition, there was a drastic reduction in the alanine turnover corrected for pool size in the dark grown experiment.

Pairwise Statistical Comparison of Pool Size and Turnover Adjusted for Pool Size

Pairwise statistical comparisons were made between each sample pair (Table S4). Many experimental pairs, despite showing appreciable graphical difference did not show statistical significance at least to the $p < 0.05$ level. This may be due to large sample variances in many of the pool size measurements. The most significant differences in pool size were present

TABLE 1 | The modeled values for turnover number (*k*) and the [15N] labeling plateau value *c* for amino acids measured in *S. polyrhiza*.

Amino acid	Experiment	*k*-value	Standard error (k)	*c*-value	Standard error (c)
Alanine (A)	Light with sucrose	0.85	0.05	0.13	0.01
	Light without sucrose	0.47	0.03	0.16	0.02
	Dark with sucrose	0.03	0.01	0.15	0.09
Arginine (R)	Light with sucrose	1.16	0.11	0.24	0.01
	Light without sucrose	0.43	0.03	0.11	0.02
	Dark with sucrose	0.25	0.03	0.30	0.03
Asparagine (N)	Light with sucrose	0.70	0.05	0.11	0.02
	Light without sucrose	0.39	0.02	0.13	0.01
	Dark with sucrose	0.32	0.02	0.29	0.01
Aspartic acid (D)	Light with sucrose	0.38	0.03	0.32	0.02
	Light without sucrose	0.11	0.02	(0.15)	0.10
	Dark with sucrose	0.12	0.04	0.59	0.08
Glutamic acid (E)	Light with sucrose	0.78	0.05	0.17	0.01
	Light without sucrose	0.32	0.10	0.44	0.06
	Dark with sucrose	–	–	–	–
Glutamine (Q)	Light with sucrose	0.44	0.027	0.41	0.01
	Light without sucrose	0.16	0.02	0.54	0.03
	Dark with sucrose	0.19	0.05	0.74	0.03
Glycine (G)	Light with sucrose	0.09	0.02	0.22	0.08
	Light without sucrose	0.17	0.09	(0.20)	0.18
	Dark with sucrose	0.04	0.02	0.80	0.06
Histidine (H)	Light with sucrose	0.44	0.03	0.41	0.01
	Light without sucrose	–	–	–	–
	Dark with sucrose	–	–	–	–
Isoleucine (I)/Leucine (L)	Light with sucrose	0.35	0.02	0.24	0.01
	Light without sucrose	0.26	0.07	0.28	0.08
	Dark with sucrose	0.04	0.002	0.07	0.02
Lysine (K)	Light with sucrose	0.1	0.01	0.34	0.05
	Light without sucrose	0.25	0.05	0.48	0.04
	Dark with sucrose	0.09	0.05	0.77	0.07
Phenylalanine (F)	Light with sucrose	0.39	0.03	0.33	0.01
	Light without sucrose	0.82	0.05	0.03	0.02
	Dark with sucrose	1.51	0.11	0.25	0.01
Proline (P)	Light with sucrose	1.17	0.07	0.03	0.01
	Light without sucrose	0.32	0.15	0.27	0.12
	Dark with sucrose	0.02	0.004	0.40	0.07
Serine (S)	Light with sucrose	0.32	0.04	0.17	0.04
	Light without sucrose	0.36	0.03	0.15	0.03
	Dark with sucrose	(0.03)	0.02	(0.45)	0.24
Threonine (T)	Light with sucrose	0.84	0.04	0.18	0.01
	Light without sucrose	0.14	0.03	0.25	0.08
	Dark with sucrose	0.06	0.005	0.54	0.03
Tryptophan (W)	Light with sucrose	0.25	0.02	0.30	0.02
	Light without sucrose	0.06	0.03	0.30	0.13
	Dark with sucrose	0.04	0.01	0.46	0.04
Tyrosine (Y)	Light with sucrose	0.16	0.021	0.37	0.04
	Light without sucrose	–	–	–	–
	Dark with sucrose	–	–	–	–
Valine (V)	Light with sucrose	0.36	0.02	0.37	0.01
	Light without sucrose	–	–	–	–
	Dark with sucrose	–	–	–	–

All values had a statistical goodness of fit of $p < 0.01$ except where otherwise noted in parentheses.

in alanine between the two light experiments and the dark grown experiment as well as asparagine between the light with sucrose and the dark grown experiment. The results in alanine are preserved in the active pool estimates, while the results for asparagine are not. Additionally, differences in the pool size for proline between the two light experiments emerge when the pool sizes are corrected for a possible active pool. The turnover rates corrected for pool size show the most significant difference in phenylalanine and asparagine between the light without sucrose and the dark grown experiment and in serine between the two light experiments. When an active pool is considered, the differences in phenylalanine become less significant while the differences in asparagine and serine remain significant.

Amino Acids Expressed Labeling Patterns That Could be Captured by Clustering Analysis. Cluster Composition Was Not Conserved When *k*-Means Clustering Was Applied to Turnover Rates Adjusted for Pool Sizes

Clustering analysis was performed using a *k*-means clustering method to assess the similarities that may be present in the label incorporation for amino acids (**Figure 5**). The appropriate number of clusters ($k_{clusters}$) was determined empirically by minimizing the within group sum-of-squares variation and maximizing the between group sum-of-squares variation. A $k_{clusters}$ of 3 or 4 was found to be most appropriate depending on the experiment. Broad patterns were seen repeated between treatments using this method. There were distinct patterns in labeling patterns that could be seen between the growth treatments. Glutamine consistently showed the fastest turnover in all experiments, closely followed by glutamate and aspartate. Both light treatments had very similar rapidly turning over clusters consisting of glutamine, glutamate, aspartate, serine, and alanine. However, this grouping is broken up in the dark treatment where glutamine maintained a rapid turnover closely followed by glutamate and aspartate while a number of amino acids involved in carbon and nitrogen central metabolism, including asparagine, serine, and alanine displayed lower turnover and clustered separately from glutamate and aspartate. Each cluster was modeled by a non-linear regression (**Table 2**).

K-means clustering was also performed using the estimated turnover rates adjusted for pool size for each amino acid individually. Exponential decay models for each amino acid and the clusters generated when considering turnover adjusted for pool size mapped onto a representation of amino acid nitrogen flow are shown in **Table 2** and **Figure 6**, respectively. Several amino acids that seemed to be relatively "core" in the previous analysis (glutamine, aspartate, glutamate, and asparagine) retained their rapid turnover. Some amino acids that grouped into apparently rapid turnover segments in the first two analyses, notably glycine and arginine in the light experiments, were shown to have much lower numbers when pool sizes were taken into consideration. This demonstrates that

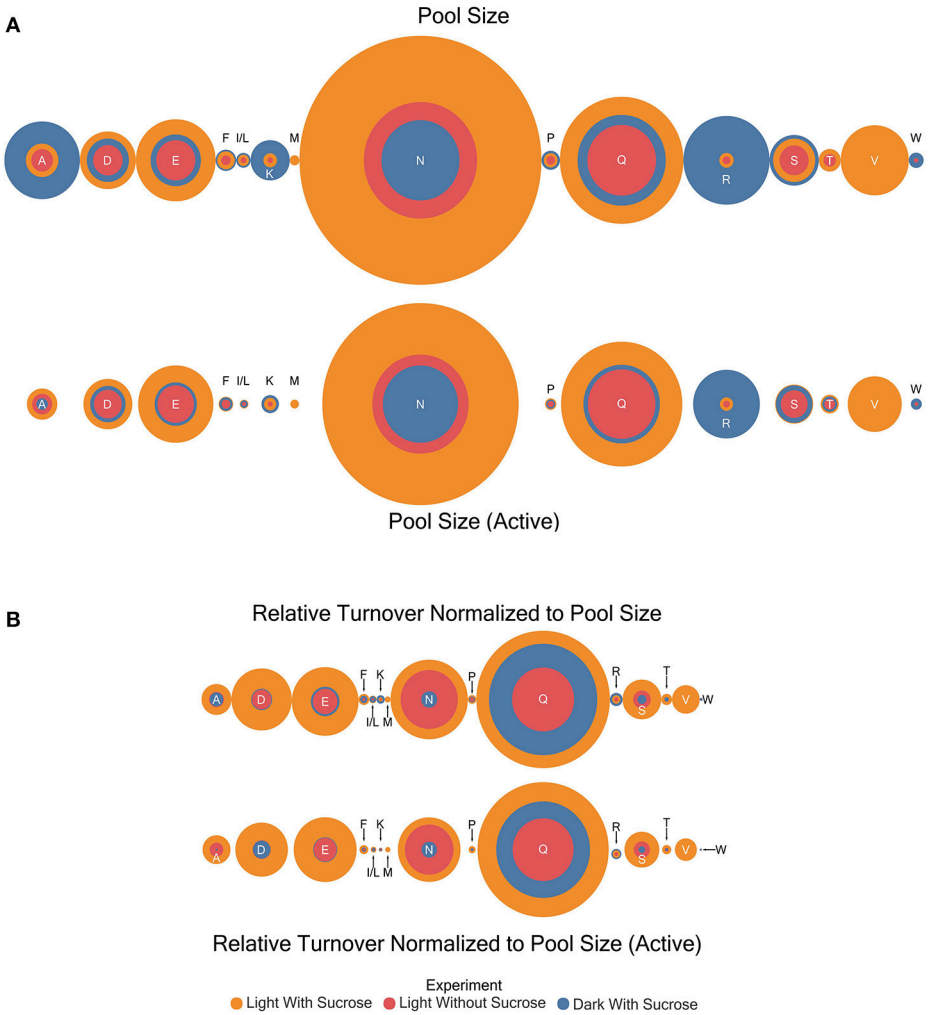

FIGURE 4 | Circle area plots showing a visual representation of relative amino acid pool sizes (μmole) and turnover correct for pool size (μmole/hr) in each experiment. Colors indicate the experiment as shown. Circle areas are proportional to the magnitude of the estimate indicated. **(A)** Relative amino acid pool size and active pool size estimates **(B)** Results for turnover normalized to pool size for total and active portions.

modeling based on the pattern of labeling can be a useful way to categorize metabolites into broad metabolic groups without needing to individually model each amino acid in turn. However, this approach may convolute some details and may also be affected to a certain extent by inactive pools or plateaus in the labeling pattern and might also not reflect a full picture of the quantity of material moving through a given metabolite.

DISCUSSION

We used stable isotope labeling to model the kinetics of amino acids grown under three different light and/or carbon source conditions. Two conditions used a light cycle, either with or without 10% w/v sucrose in the growth media and one condition grown in the dark with 10% w/v sucrose in the growth medium. Growing an angiosperm plant under these three different conditions adds a considerable amount to the existing body of research involving stable isotope labeling *in planta* (Freund and Hegeman, 2017). Previous studies involving the labeling of whole plants generally have involved plants grown exclusively in heterotrophic conditions (Kikuchi et al., 2004; Szecowka et al., 2013). Photoautotrophic, mixotrophic, and heterotrophic growth conditions have all been investigated in the microalgae *Chlamydomonas reinhardtii* (Boyle and Morgan, 2009), however, there is a limited number of studies investigating whole plants grown and labeled under these three conditions. In particular we lay the groundwork for future intensive labeling studies for *S. polyrhiza* by demonstrating experimental and analytical frameworks that can be adapted to [^{13}C]-labeling through the use of [^{13}C]O_2 or [^{13}C]-labeled sugars.

The three growth conditions used in this study reflect fundamentally different metabolic modes, which in turn has an affect on the growth of the plant. This difference in plant status can be seen at both macroscopic and microscopic levels

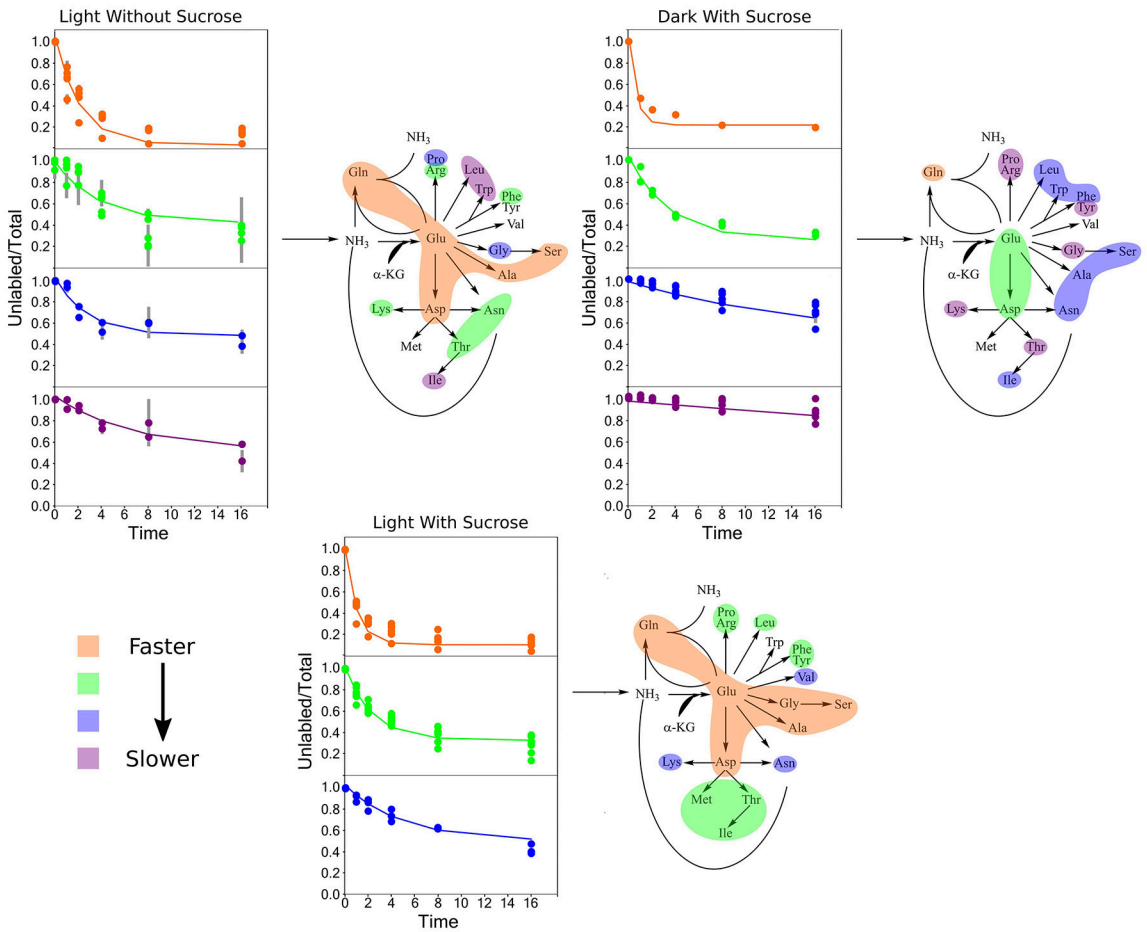

FIGURE 5 | Average labeling patterns following the decay of the unlabeled isotopomer over time for each experiment. The lines are the models generated by *R* and the error bars represent ±1 SE. Maps adjacent to each set of graphs represent the flow of nitrogen among amino acids. The colored bubbles correspond to the clusters in the above figure highlighting similar isotopic exchange rates for the members of a cluster.

(**Figure 2**) as plants grown in light show normal coloration and chlorophyll autofluorescence while those grown in the dark show a bleached color and lack any sign of chlorophyll autofluorescence while still retaining plastid structures.

Labeling kinetics of each individual amino acid also shows interesting patterns. After analyzing and inspecting the data we chose to model the decay of the unlabeled isotopomer following Yuan et al. (2006) with an added term to account for the visible plateau reached by almost all of the amino acids by about 16 h. Each of these models, with few exceptions, has a very high goodness of fit level ($p < 0.001$) despite somewhat large standard errors in some cases. Though as noted in a similar analysis carried out by Szecowka et al. (2013) this may be due to an averaging effect across compartments, which could not be resolved in our current analysis. As described above models were generated using the time 0–16 h points for almost all amino acids. However, for certain amino acids, most notably many in the dark grown experiment the apparent plateau was not fully realized until closer to the 64 h time point, if at all.

TABLE 2 | A summary of the exponential decay models for each cluster.

Experiment	Cluster	*k*-value	p (k)	SE	c (constant)	SE	p (c)
Light	1	1.002	***	0.05	0.179	0.01	***
With	2	0.437	***	0.02	0.380	0.01	***
Sucrose	3	0.205	***	0.03	0.530	0.04	***
	1	0.472	***	0.04	0.110	0.03	***
Light	2	0.128	***	0.05	0.000	0.25	–
Without	3	0.360	***	0.10	0.529	0.06	***
Sucrose	4	0.149	–	0.08	0.547	0.15	***
	1	1.659	***	0.12	0.278	0.02	***
Dark	2	0.286	***	0.03	0.302	0.03	***
With	3	0.061	**	0.03	0.514	0.19	***
Sucrose	4	0.008	–	0.08	0.000	9.26	–

*Statistical significance is indicated by asterisks with the following levels represented: ***$p < 0.005$, **$p < 0.01$, and $^{-}p > 0.05$.*

This failure to reach a plateau can be seen in **Figure 7** for glutamine. Both light experiments have models and labeling patterns that follow each other closely. However, the dark grown

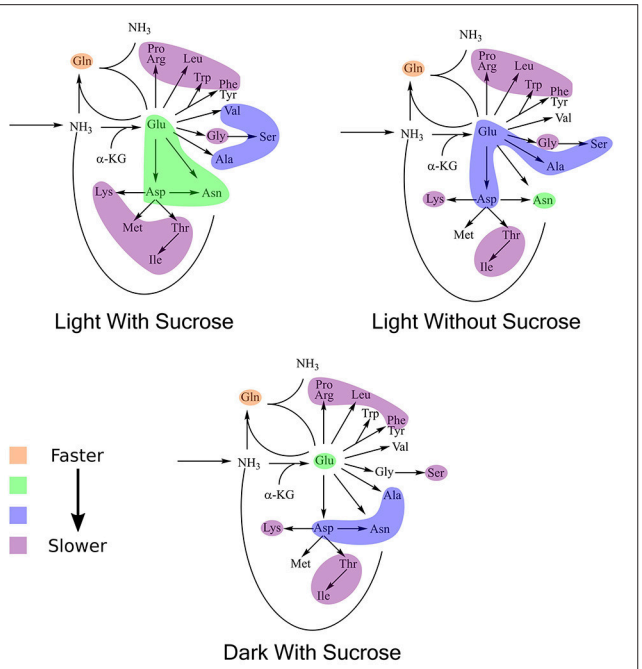

FIGURE 6 | Results of *k*-means clustering for estimates of turnover normalized to active pool size. Colors of each bubble represent the same cluster positions (1–4) and relative exchange rates as in **Figure 5**.

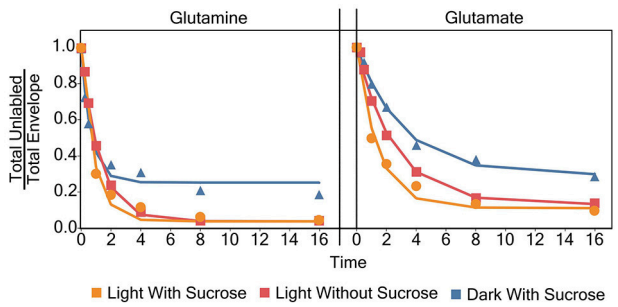

FIGURE 7 | Individual models for glutamate and glutamine for each experiment.

experiment begins to deviate from the model after the 16 h time point. This pattern continues for several amino acids in the dark grown condition, including: aspartate, glutamate, phenylalanine, isoleucine/leucine, lysine, proline, and serine. There are several possibilities that could account for this deviation from the initial models seen in the dark grown samples. Rather than a plateau/inactive pool, it would be simplest to model a two-phase labeling. This model would consist of an initial (fast) labeling, similar to our initial estimate followed by a slower second phase as (1) the larger-than-estimated active pool is completely washed out or (2) certain sub-pools/compartments with lower turnover begin to take up label. In addition, some irregularities can be observed in the labeling patterns, again in **Figure 7**, and as discussed later regarding metabolic clusters, where glutamine appears to label at a faster rate than glutamate, which seems novel as glutamate is the first entry point for nitrogen during uptake. It is uncertain what causes this labeling pattern, but it is possible that glutamine, which possesses multiple nitrogen atoms, may exhibit two different turnover coefficients, causing one nitrogen atom, most likely in the side chain, to label much more slowly than the alpha nitrogen.

We also used comparison of the time zero samples to a labeled standard to achieve estimates of pool size for most of the amino acids measured. No similar estimates have been carried out in any duckweed species to date to our knowledge making this a unique and useful data set. Several clear patterns emerged in these pool size estimates including the dark experiment showing larger pool sizes in alanine, phenylalanine, isoleucine/leucine, lysine, arginine, serine, threonine, and tryptophan. This result is notable, as many of these amino acids have been shown to accumulate in chlorophyll-deficient mutants during recovery of a normal pigment phenotype (Maclachlan and Zalik, 1963; Shortess and Amby, 1979). That we see the same patterns between dark grown and light grown with sucrose as was previously seen with some chlorophyll mutants indicates that the differences we are observing likely do come from a difference in autotrophic vs. heterotrophic status of the cell.

In addition to estimating total pool size, we also estimated an active pool size based on the observed plateaus in each labeling pattern. These active pools were defined as the total pool size minus a part of the pool size proportional to the modeled plateau. When this estimation was taken into account many of the amino acids displaying larger pool sizes in the dark no longer displayed larger pool sizes with respect to the light with sucrose treatment, with the exception of lysine, arginine, and serine. This result shows larger inactive pools, overall, in the dark grown experiments. This is significant particularly in asparagine, which is known to be a particularly prevalent nitrogen storage molecule (Lea et al., 2007), and one that is sensitive to the carbon status of the plant as well as arginine, which is generally a primary nitrogen storage molecule in the roots (Winter et al., 2015). As arginine pool sizes seem largest in the dark this could be an indication that metabolism in dark grown duckweed is functioning more like sink tissues such as roots.

Turnover rates were normalized to pool size by multiplying the rate by pool size estimates for each amino acid. By making this adjustment we are able to estimate a quantity with the dimensions of a flux parameter (moles/unit time) that can help better inform the results that we observed in the labeling patterns. While the turnover rates for many amino acids were noticeably slower in the dark, we can see that relatively larger pool sizes of these samples result in more moderate differences between treatments when considering the pool size normalized turnover rate values. This is consistent with the idea that those plants have a much more robust nutrient supply having carbon available through both photosynthetic carbon fixation and through supplemental sucrose in the medium. The notable exceptions to this are arginine and lysine which both show higher pool size adjusted turnover values in the dark. These pool size adjusted turnover rates were also calculated for the estimated active pools and yielded similar results. The

one exception is the large decrease in pool size normalized turnover rate when estimated with the active pool size in alanine.

One of the most biologically interesting results is that of serine and the high level of statistical significance found for the difference between pool size adjusted turnover rate in the light experiments with and without sucrose and also between the light without sucrose and dark grown experiments. This is an even more notable result because of a recent interest in serine metabolism. The main source of serine biosynthesis in most conditions is the glycolate pathway (Douce et al., 2001; Ros et al., 2014). This pathway is associated with photorespiration and is thus not expected to be a contributor to serine turnover in conditions where photorespiration is low or nonexistent, such as in a root or an etiolated plant as in the dark grown condition potentially leading to lower turnover overall.

Before attempting individual modeling of amino acids we employed clustering analysis to identify broad patterns in amino acid labeling without the need to calculate individual models. This met with some success. However, when investigating a metabolic system, the most important measure is going to be the pathway flux. While we have not estimated a true flux through each amino acid for reasons outlined above pool size adjusted turnover rates give us a measurement with the dimensions of flux. When similar clustering analysis is carried out on these normalized rate values general patterns are still observed. However, many clusters now contain only one metabolite giving far less meaningful results. Even with this many of the patterns that are seen in the turnover adjusted for pool size, especially areas of relatively rapid turnover can still be seen. Though, there are some notable changes such as asparagine showing considerably higher turnover than was estimated in the light with sucrose condition through labeling pattern alone. Thus, while useful, due to the fact that they cannot fully capture fluxes and other more quantitative information a clustering-type analysis may be more useful when attempting to investigate metabolic dynamics where full identification and quantification of the involved metabolites is not possible, such as with portions of secondary or specialized metabolism.

CONCLUSION

In this study stable isotope labeling was used to investigate labeling patterns, pool sizes, and turnover in *S. polyrhiza* under three growth conditions corresponding, roughly to full photoautotrophic growth, mixotrophic growth, and full heterotrophic growth and demonstrated growth of *S. polyrhiza* in the dark for the first time as well as providing the first amino acid pool size estimates for *S. polyrhiza*. These data established the clear labeling plateau and likely presence of metabolically inactive or less active pools in experiments with a light cycle. They also showed that correcting for active pool sizes could, in some cases, have a large effect on comparisons between experiments. We showed that pool sizes are not sufficient to make full metabolic comparisons and that adding a flux dimension, whether via full metabolic flux analysis or the use of turnover number corrected for pool size, can highlight differences that would be otherwise missed in simply considering pool size or turnover number alone.

Labeling patterns in each condition highlighted some interesting known (asparagine pool sizes in the dark) and emerging (serine turnover adjusted pool sizes) trends, particularly, the results regarding serine support the function of photorespiratory pathways as the most efficient/highest turnover mechanism of serine metabolism as we see pool accumulation in conjunction with low turnover in heterotrophic growth conditions. Labeling patterns were also captured by *k*-means clustering analysis. When pool sizes and turnover numbers were considered it became clear that many differences apparent when using those metrics could be masked when only considering the labeling pattern. However, the agreement that does exist between labeling pattern-derived clusters and turnover normalized for pool size indicate that application of clustering techniques based on labeling pattern could be useful for situations where many compounds are unknown, such as investigation of secondary metabolism.

DATA AVAILABILITY

The datasets generated and analyzed for this study can be found in University of Minnesota Digital Conservancy Data Repository: http://hdl.handle.net/11299/193599.

AUTHOR CONTRIBUTIONS

EE wrote the manuscript and was primarily responsible for carrying out labeling experiments, sample collection, LC-MS analysis, and data analysis. DF assisted in design of labeling experiments and provided the LC-MS method. DF and VS assisted in carrying out labeling experiments and in LC-MS data collection. VS assisted in data analysis. JC and AH provided funding and project direction. All authors read, edited, and approved the manuscript.

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Roden for the design of the turnover and clustering *R* scripts that were used. This work also appears in a MS thesis submitted by EE and is deposited in the University of Minnesota Digital Conservancy, as is consistent with University of Minnesota policy.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00191/full#supplementary-material

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Correlation of Apiose Levels and Growth Rates in Duckweeds

Débora Pagliuso[1], *Adriana Grandis*[1], *Eglee S. Igarashi*[1], *Eric Lam*[2]* and *Marcos S. Buckeridge*[1]*

[1] Laboratory of Plant Physiological Ecology, Department of Botany, Systems and Synthetic Biology Center, Institute of Biosciences, University of São Paulo, São Paulo, Brazil, [2] Department of Plant Biology, Rutgers, The State University of New Jersey, New Brunswick, NJ, United States

The carbon assimilated by photosynthesis in plants can be partitioned into starch, soluble sugars, and cell wall polymers. Higher levels of starch accumulation in leaves are usually correlated with a lower growth capacity. Duckweeds are fast-growing aquatic monocot plants that can accumulate high levels of starch. They are an unusual group because their cell wall has very low levels of lignin while accumulating apiogalacturonan, a pectic polysaccharide that could be involved with boron assimilation. In this work, five duckweed species from different genera (*Spirodela polyrhiza*, *Landoltia punctata*, *Lemna gibba*, *Wolffiella caudata*, and *Wolffia borealis*) were cultivated under two light intensities (20 and 500 μmoles of photons m^{-2} s^{-1}) to evaluate the effects of growth rate on carbohydrate metabolism. A comparative analysis was performed by measuring their relative growth rates (RGR), and their content for starch, as well as soluble and cell wall carbohydrates. We found that the faster-growing species (the Lemnoideae) accumulate lower starch and higher soluble sugars than the slower-growing species within the Wolffioideae. Interestingly, analysis of the cell wall monosaccharides revealed that the slower-growing species displayed lower content of apiose in their walls. Our results indicate that higher accumulation of apiose observed in cell walls of the Lemnoideae species, which likely correlates with a higher proportion of apiogalacturonan, may lead to higher efficiency in the assimilation of boron. This is consistent with the increased RGR observed under conditions with higher apiose in the cell wall, such as higher light intensity. Consistent with their lower growth capacity, the Wolffioideae species we studied shows higher starch accumulation in comparison with the Lemnoideae species. We suggest that apiose levels could be good biomarkers for growth capacity of duckweeds and suggest that boron uptake could be an important factor for growth control in this aquatic plant family.

Keywords: duckweed, lemnoideae, wolffioideae, starch, cell wall, apiose, growth

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***Correspondence:**
Eric Lam
ericl89@hotmail.com
Marcos S. Buckeridge
msbuck@usp.br

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INTRODUCTION

Duckweeds (the family Lemnaceae) are the smallest monocots and live as free-floating aquatic plants (Landolt, 1992; Appenroth et al., 2013). The 37 species of Lemnaceae have been classified into five genera (*Spirodela, Landoltia, Lemna, Wolffiella,* and *Wolffia*) based on their morphology and physiology (Borisjuk et al., 2015). They are further subdivided into two subfamilies, the Lemnoideae (*Spirodela, Landoltia,* and *Lemna*) and Wolffioideae (*Wolffiella* and *Wolffia*) (Les et al., 2002), the latter being the rootless duckweeds. Fast-growing and starch accumulation capacities are some of

the main features of duckweed, with some strains having been shown to double in biomass within 96 h (10 times faster than maize) (Yu et al., 2014).

Plant growth depends on carbon assimilation through photosynthesis. During the day, starch is synthesized and stored in the plastids, whereas sucrose is stored in vacuoles or directly used for growth. In the dark, the stored starch will serve as the main compound to support plant growth (Mengin et al., 2017). Thus, starch contents vary according to photoperiod (Smith and Stitt, 2007; Zeeman et al., 2007, 2010; Fernandez et al., 2017). Yin et al. (2015) found positive correlations between day length, light intensity, and level of starch accumulation in *Lemna aequinoctialis*. Starch levels are also found to be related to the nutrient status (Xiao et al., 2013). These authors found that growth of *Landoltia punctata*, *Spirodela polyrhiza*, and *L. aequinoctialis* is boosted with a concomitant decrease of starch under higher availability of P and N. Thus, lower concentrations of starch can be a sign for higher growth rates and vice-versa.

In plants, most of the carbon assimilated by photosynthesis is partitioned into cell walls (Vaughan et al., 1992; Verbančič et al., 2017). Plant cell walls form a Glycomic Code (Buckeridge, 2018) that may help to determine their structure-function relationship. The cell wall is composed of polysaccharides, phenolic compounds, and proteins, the former being quantitatively more dominant. Besides providing mechanical support for plant tissues, the cell wall also acts as a defense mechanism by presenting a physical barrier to biological invaders (Sarkar et al., 2009; Kalluri and Keller, 2010). In cell walls, cellulose microfibrils are the architectural core to which hemicelluloses (xyloglucan, arabinoxylans, mannans, beta-glucans, and others) are attached. This domain (cellulose-hemicellulose) is immersed in a matrix of pectins that include homogalacturonans and rhamnogalacturonans, branched with neutral chains of galactans, arabinans, and arabinogalactans (Carpita and Gibeaut, 1993). In this regard, the pectin domain of many duckweeds is unique. Besides rhamnogalacturonans and arabinogalactans (Venketachalam et al., 2013), many species of the Lemnaceae have been found to have cell walls enriched with apiogalacturonan, a pectin polymer rich in apiose (Hart and Kindel, 1970; Mølhøj et al., 2003; O'Neill et al., 2004; Camacho-Cristóbal et al., 2008; Miwa and Fujiwara, 2010; Bar-Peled and O'Neill, 2011). Recently, Avci et al. (2018) observed that pectins in Lemnoideae are mainly apiogalacturonans, whereas in Wolffioideae the apiogalacturonan content is reduced, and replaced by xylogalacturonan. The authors highlighted a possible evolutionary trend in duckweeds associated with species-dependent variations in apiogalacturonan and xylogalacturonan (Avci et al., 2018). Apiose-containing polysaccharides are thought to play a role in the boron binding capacity of duckweeds (Matoh and Kobayashi, 1998) as well as to plant development and growth (Blevins and Lukaszewski, 1998; Matoh and Kobayashi, 1998). Relatively little is known about the other cell wall polymers of duckweeds (Zhao et al., 2014). Hemicelluloses have been reported to be in small amounts (3%) in duckweed cell walls (Ge et al., 2012; Zhao et al., 2014), consistent with the presence of very low lignin levels in duckweed biomass (Blazey and McClure, 1968). Venketachalam et al. (2013) identified xyloglucans and xylans in the glycome profile of *Lemna*, while cellulose has been reported to be present at 43.7% in *Lemna minor* cell walls (Zhao et al., 2014)

In this work, we performed a comparative analysis of growth rates and carbohydrate contents under different light conditions with five species of duckweed from the different genera. We found that under higher light intensity, a condition in which the RGR increases in all species, the faster-growing Lemnoideae species accumulate less starch than the slower-growing Wolffioideae species. At the same time, the higher light intensity increased growth as well as the proportion of apiose in the cell walls of Wolffioideae. Our results suggest that the presence of apiose-containing polymers in cell walls of duckweeds could be related to the growth capacity of duckweed species.

MATERIALS AND METHODS

Plant Material, Cultivation, and Sample Preparation

Spirodela polyrhiza (9509), *Landoltia punctata* (7624), *Lemna gibba* (DWC128), *Wolffiella caudata* (9139), and *Wolffia borealis* (9144) were obtained from the Rutgers Duckweed Stock Cooperative (RDSC) collection (**Figure 1**). Duckweeds were cultivated under axenic conditions in 100 mL of ½ Schenk-Hildebrandt medium (pH 6.5) with 0.5% of sucrose. The plants were grown at 25°C with a photoperiod of 16 h of light. Two different light intensities were used: 20 μmoles m^{-2} s^{-1} and 500 μmoles m^{-2} s^{-1}. The cultivated plants were frozen in liquid nitrogen and freeze-dried. The freeze-dried samples were transferred to 15 mL polycarbonate vials with the screw-on cap including a steel grinding ball of 11 mm that was placed in Geno/Grinder®2010 SPEX SamplePrep for sample processing at 1400 rpm for 2 min or until a fine powder achieved.

Relative Growth Rate Measurements

The RGR parameters were calculated according to International Steering Committee on Duckweed Research and Applications (ISCDRA). The growth measurements followed the procedure described by Ziegler et al. (2015). Twenty fronds of *S. polyrhiza*, *L. punctata, L. gibba,* and 50 fronds from *W. caudata* and *W. borealis* were initially inoculated into the culture medium. At the same time, for T_0 (the initial point of analysis) four replicates were harvested for measurements of dry mass. T_7 was harvested after 7 days of growth. Samples were dried at 105°C for 12 h and weighed to obtain dry mass. RGR was calculated by equation I that was simplified into equation II, where x represents the data of the evaluated parameters (dry mass and number of fronds) and t represents elapsed time (zero -t_0-and 7 days-t_7).

$$X_t = x_{t0} * e^{RGR * t} \qquad (I)$$

$$RGR = \frac{lnx_{t7} - lnx_{t0}}{t_7 - t_0} \qquad (II)$$

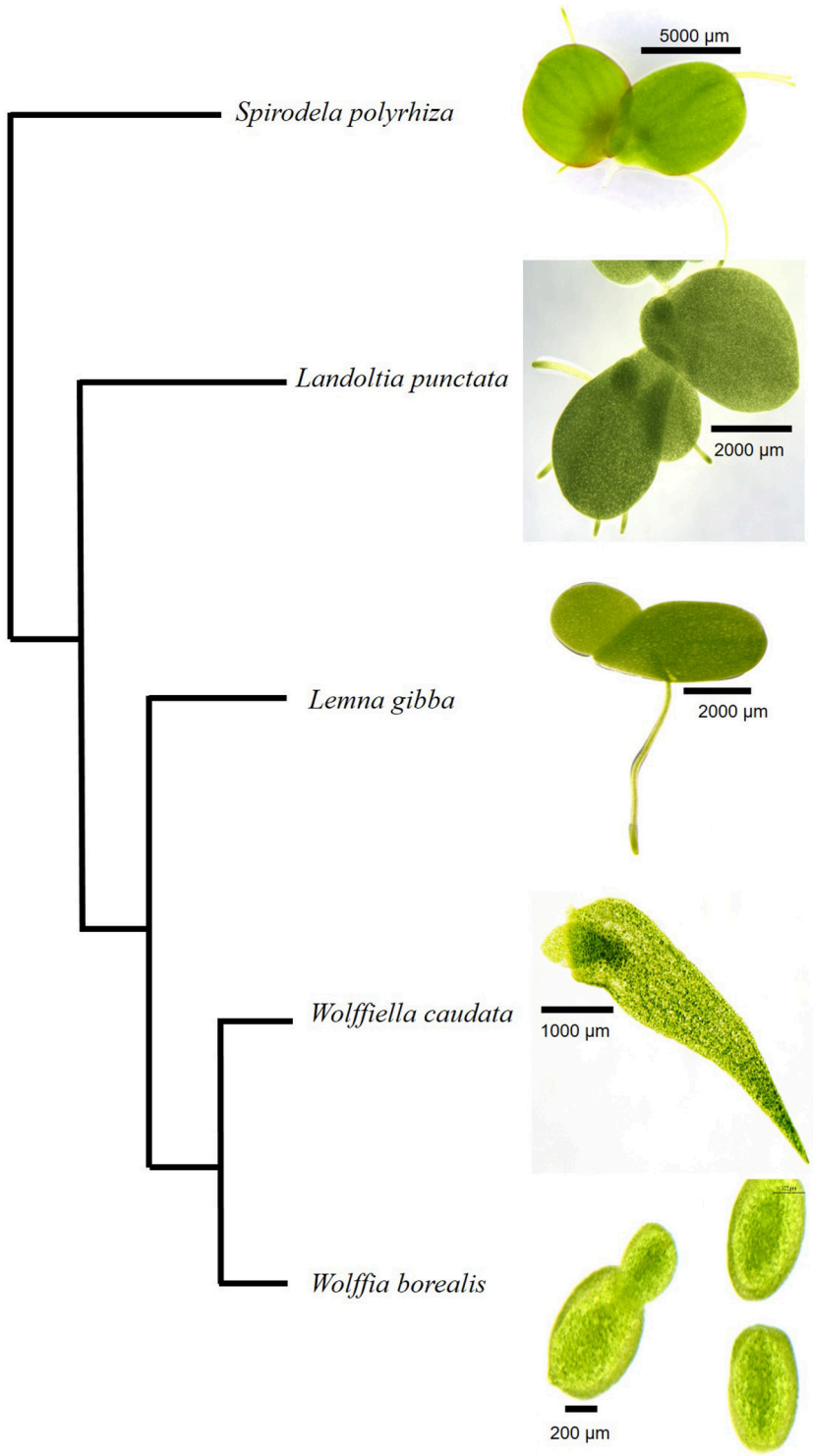

FIGURE 1 | Cladogram of *Lemnaceae* species analyzed in the present study. The five species from different genera of duckweeds are shown according to the phylogeny suggested by Tippery et al. (2015). A size bar in each photograph indicates the scale as shown for the different specimen.

Soluble Carbohydrate Extraction and Analysis

Soluble sugars (glucose, fructose, sucrose, and raffinose) were extracted four times from 20 mg (dry mass) of pulverized samples with 1.5 mL of 80% ethanol at 80°C for 20 min. The Alcohol Insoluble Residue (AIR) was dried at 45°C overnight. The supernatant was recovered, vacuum concentrated (ThermoScientific® Savant SC 250 EXP) and resuspended in 1 mL of water and 1 mL of chloroform. The soluble sugars (sucrose, fructose, glucose, and raffinose) were analyzed by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) in a Dionex® system (ICS 5000) using a CarboPac PA1 column and eluted with 150 μM sodium hydroxide in an isocratic run of 27 min (Supplementary Figures 1, 2).

Starch Removal and Determination

Starch was measured according to Amaral et al. (2007) and Arenque et al. (2014). AIR was treated with 120 U/mL of α-amylase (E.C. 3.2.1.1) of *Bacillus licheniformis* (Megazyme® Inc., Australia) diluted in 10 mM MOPS buffer pH 6.5 at 75°C for 1 h. Incubation was followed by addition of 30 U/mL of amyloglucosidase (E.C. 3.2.1.3) of *Aspergillus niger* (Megazyme® Inc., Australia) diluted in 100 mM sodium acetate pH 4.5 at 50°C for 1 h. The reactions were stopped by freezing the samples. The supernatants were recovered by centrifugation, and the pellets were washed three times with 80% ethanol and dried at 45°C overnight and reserved for monosaccharides analysis. For starch determination, 5 μL of each sample was diluted with 45 μL of deionized water followed by 250 μL of a mixture containing glucose oxidase (1,100 U/mL), peroxidase (700 U/mL), 4-aminoantipirin (290 μmol/L) and 50 mM of phenol at pH 7.5. The plates were incubated for 15 min at 30°C and the absorbance was measured at 490 nm. The calibration curve was performed with commercial glucose (Sigma®) in the concentration range of 0.02–0.2 mg/mL.

Monosaccharide Composition

Five mg of the cell wall (de-starched AIR) was hydrolyzed with 1 mL of 2 M trifluoroacetic acid (TFA) for 1 h at 100°C. The reaction mixture was dried under vacuum and resuspended in 1 mL of deionized water. This was followed by filtration on 0.22 μm (Merck Millipore®) filters. The released monosaccharides were analyzed by HPAEC-PAD through the injection of 10 μL hydrolysate into a CarboPac SA10 column (ICS 5.000 system, Dionex-Thermo®). The column was eluted isocratically with 99.2% of water and 0.8% (v/v) sodium hydroxide (1 mL/min). The monosaccharide release from the cell wall were detected using a post-column base containing 500 mM NaOH (0.5 mL/min). The standards used were apiose, arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose (Supplementary Figure 1). Quantification was performed by injections of samples with known concentrations for each monosaccharide to calibrate the instrument.

Uronic Acid Determination

The total uronic acid was quantified according to Filisetti-Cozzi and Carpita (1991). Five mg of each de-starched cell wall were weighed and 2 mL of concentrated sulfuric acid were added. The reactions were incubated for 10 min on ice under stirring (1,250 rpm), followed by addition of 1 mL of deionized water. This procedure was repeated once. The incubated mixtures were diluted to 10 mL and centrifuged at 4,000 g for 10 min at room temperature. Forty μL of 4 M sulfamic acid/potassium sulfamate solution (pH 1.6) and 2.4 mL of 75 mM sodium borate in sulfuric acid was added to aliquots of supernatant (400 μL). The homogenized solutions were incubated at a 100°C for 20 min, then cooled on ice for 10 min. Eighty microliter of m-hydroxybiphenyl in 0.5% NaOH were added and vortexed for color development. The samples were read at 525 nm in Spectrophotometer Genesys 10S UV-VIS ThermoScientific®. A standard curve using D-galacturonic acid was performed in the concentration range of 5–40 μL/400 μL.

Statistical Analysis

Four replicates were used for the experiments. Interspecific analyses were performed by ANOVA one-way followed by Tukey's test ($p < 0.05$). A *t*-test was used to compare different light conditions. The analyses were carried out using JMP® software version 5.1 or R version 3.2.2. Principal Component Analysis was performed using Minitab software version 14 with all data for low light and high light treatments. General Linear Model (GLM) was used to evaluate the significance of each principal component (Supplementary Table 2).

RESULTS

Relative Growth Rates

We first compared the RGR between the five species of duckweed under illumination at intensity of 20 μmoles m^{-2} s^{-1}. Using either dry weight (RGR-D, **Figure 2A**) or frond number (RGR-F, **Figure 2B**) as the basis for RGR calculation, we found that the rates for the two Wolffioideae species (*W. caudata* and *W. borealis*) were significantly lower (0.1–0.14 day^{-1}) than the other species of the Lemnoideae (0.14–0.24 day^{-1}). In contrast, when compared under higher light intensity of 500 μmoles m^{-2} s^{-1}, RGR-F measurements continue to show lower growth rates for the Wolffioideae (**Figure 2D**) while RGR-D measurements showed equally high growth rates for all five species of duckweed (**Figure 2C**).

Quantifying the effects of light intensity on these parameters for each species revealed that RGR-D increased the most for the Wolffioideae under higher light intensities, being at around 50% for *S. polyrhiza* (53.9%) and *L. punctata* (42.7%) and more than doubling for *L. gibba* (147.9%), *W. caudata* (203.4%), and tripling for *W. borealis* (218.6%). Since the frond number increase (RGR-F) in the Wolffioideae continues to be below that of the Lemnoideae (**Figure 2D**), our results indicate that the fronds of Wolffioideae produced under higher light must be higher in dry mass than the Lemnoideae fronds, thus resulting in

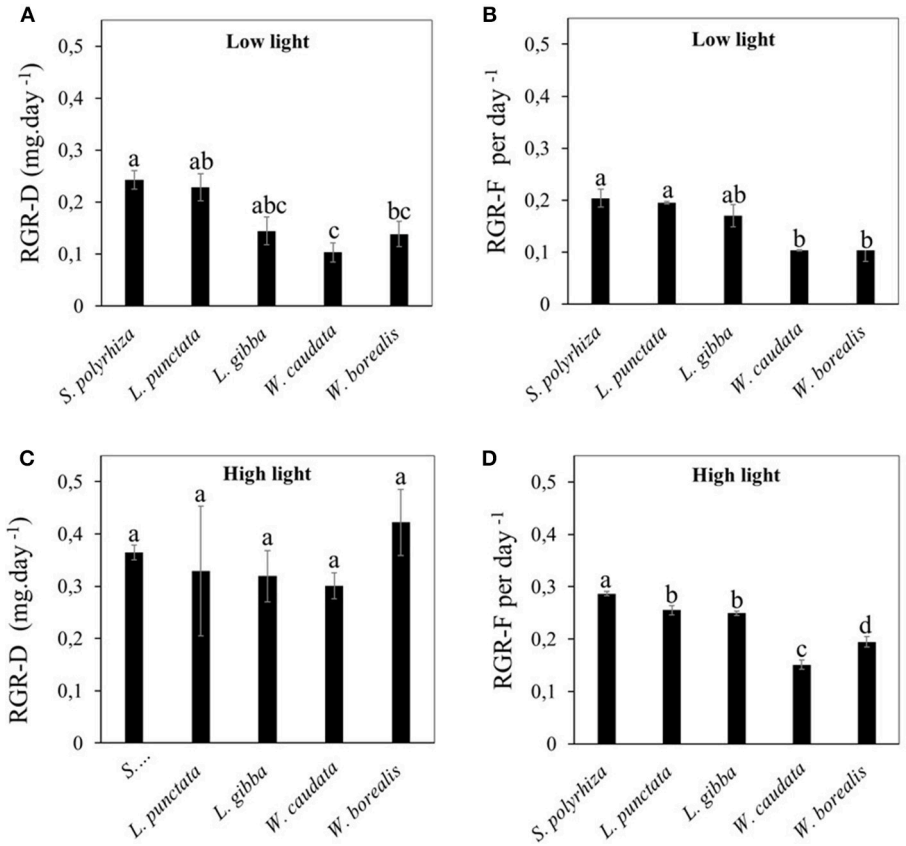

FIGURE 2 | Comparison of relative growth rate (RGR) for different duckweed strains (*S. polyrhiza, L. punctata, L. gibba, W. caudata*, and *W. borealis*) cultivated under two light intensities (20 μmol.m^{-2}.s^{-1} and 500 μmol.m^{-2}.s^{-1}). Values are average ± standard error evaluated per dry mass **(A,C)** and the number of fronds **(B,D)**. **(A,B)** show data from low light condition while **(C,D)** represent data from high light condition. Means followed by the same letters are statistically significant among the five species compared according to Tukey's test ($p < 0.05$).

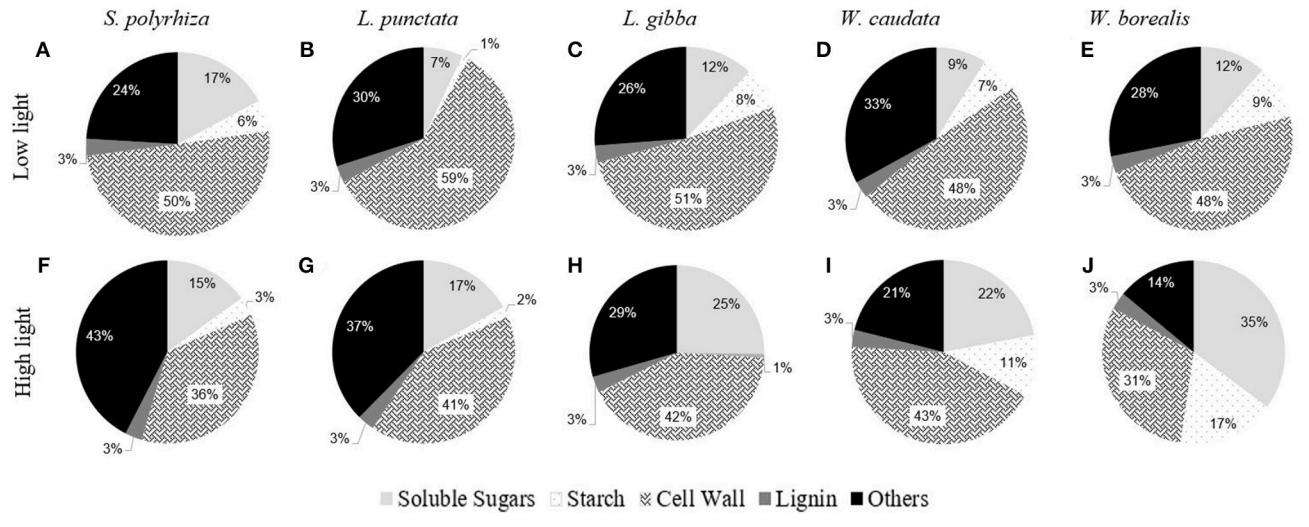

FIGURE 3 | Comparative composition of duckweeds (*S. polyrhiza, L. punctata, L. gibba, W. caudata*, and *W. borealis*) biomass cultivated under low light (20 μmol.m^{-2}.s^{-1}) and high light (500 μmol.m^{-2}.s^{-1}) intensity. Values are averages from the percentages of dry mass. Soluble sugars are the sum of the contents of sucrose, fructose, raffinose, and glucose. Cell walls are the residual portion of de-starched AIR. ($n = 4$). The capital letters represent the species and the light treatment. **(A–E)** are the biomass composition of plants cultivated under low light and **(F–J)** under high light.

TABLE 1 | Sugar composition of duckweeds (*S. polyrhiza, L. punctata, L. gibba, W. caudata*, and *W. borealis*) biomass ($\mu g.mg^{-1}$ dry mass) cultivated under low light (20 $\mu mol.m^{-2}.s^{-1}$) (LL) and high light (500 $\mu mol.m^{-2}.s^{-1}$) (HL) intensities.

		S. polyrhiza	*L. punctata*	*L. gibba*	*W. caudata*	*W. borealis*
NON-STRUCTURAL CARBOHYDRATES ($\mu g.mg^{-1}$ DM)						
Glucose	LL	5.03 ± 2.11	2.64 ± 0.40	6.10 ± 2.19	4.00 ± 0.45	5.12 ± 1.04
	HL	2.66 ± 0.19	1.63 ± 0.17	4.54 ± 0.55	2.40 ± 0.25	12.38 ± 4.84
	P-value	0.306	0.061	0.515	**0.020**	0.193
Fructose	LL	6.51 ± 3.01	4.33 ± 0.72	5.67 ± 2.14	3.99 ± 0.41	6.37 ± 1.19
	HL	2.89 ± 0.32	1.84 ± 0.19	5.80 ± 0.72	5.19 ± 0.87	17.03 ± 5.83
	P-value	0.276	**0.015**	0.953	0.259	0.124
Sucrose	LL	5.47 ± 2.21	0.04 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.17 ± 0.02
	HL	8.81 ± 0.65	6.48 ± 0.50	11.02 ± 0.58	14.26 ± 2.25	5.66 ± 0.22
	P-value	0.198	**0.000**	**0.000**	**0.001**	**0.000**
Raffinose	LL	0.22 ± 0.10	0.01 ± 0.01	0.21 ± 0.11	0.89 ± 0.12	0.02 ± 0.01
	HL	0.68 ± 0.08	6.90 ± 0.51	3.85 ± 0.53	0.19 ± 0.06	0.12 ± 0.03
	P-value	**0.011**	**0.000**	**0.001**	**0.002**	**0.018**
Starch	LL	55.23 ± 13.88	11.97 ± 0.69	74.04 ± 23.61	66.49 ± 18.77	94.71 ± 21.94
	HL	32.95 ± 1.98	18.56 ± 3.19	6.88 ± 0.21	106.88 ± 20.61	171.54 ± 27.67
	P-value	0.163	0.090	**0.029**	0.198	0.073
STRUCTURAL CARBOHYDRATES ($\mu g.mg^{-1}$ DM)						
Uronic acids	LL	184.96 ± 17.64	66.98 ± 5.85	169.86 ± 18.18	180.36 ± 17.53	226.82 ± 33.34
	HL	127.57 ± 12.73	174.51 ± 18.79	143.77 ± 16.41	140.65 ± 36.66	123.24 ± 14.33
	P-value	**0.039**	**0.002**	0.061	0.045	0.147
Fucose	LL	1.54 ± 0.12	1.19 ± 0.21	0.98 ± 0.06	1.06 ± 0.06	1.42 ± 0.16
	HL	1.14 ± 0.06	0.53 ± 0.13	0.67 ± 0.02	1.25 ± 0.25	1.74 ± 0.27
	P-value	**0.026**	**0.040**	**0.003**	0.497	0.343
Apiose	LL	7.27 ± 0.90	19.47 ± 3.61	15.80 ± 0.75	3.61 ± 0.71	1.94 ± 0.27
	HL	5.42 ± 0.34	14.64 ± 0.72	10.16 ± 1.13	7.00 ± 1.85	3.23 ± 0.36
	P-value	0.103	0.238	**0.006**	0.139	**0.030**
Xylose	LL	8.47 ± 0.38	23.20 ± 4.48	18.26 ± 0.73	13.92 ± 0.78	15.98 ± 0.65
	HL	6.94 ± 0.31	12.63 ± 4.03	14.57 ± 1.86	24.33 ± 5.39	27.19 ± 4.20
	P-value	**0.020**	0.130	0.115	0.104	**0.039**
Arabinose	LL	11.57 ± 1.02	10.34 ± 1.91	6.17 ± 0.15	28.76 ± 4.72	14.46 ± 0.65
	HL	9.78 ± 0.71	4.03 ± 1.10	5.64 ± 0.48	39.05 ±7.02	23.80 ± 3.23
	P-value	0.203	**0.028**	0.336	0.270	**0.030**
Galactose	LL	13.80 ± 0.77	12.78 ± 2.62	8.91 ± 0.20	9.96 ± 0.89	8.68 ± 0.73
	HL	11.63 ± 0.81	6.96 ± 2.22	8.35 ± 0.61	11.60 ± 2.19	11.61 ± 1.87
	P-value	0.101	0.142	0.420	0.516	0.196
Rhamnose	LL	3.88 ± 0.24	3.26 ± 0.70	2.36 ± 0.08	1.72 ± 0.14	2.40 ± 0.21
	HL	3.23 ± 0.18	1.69 ± 0.53	1.88 ± 0.17	2.31 ± 0.58	2.37 ± 0.33
	P-value	0.073	0.124	**0.050**	0.364	0.950
Glucose	LL	3.39 ± 0.44	3.05 ± 0.45	3.02 ± 0.71	5.07 ± 0.81	4.74 ± 1.46
	HL	9.02 ± 1.08	2.96 ± 0.92	4.76 ±1.30	14.27 ± 3.49	17.96 ± 3.95
	P-value	**0.003**	0.932	0.284	**0.043**	**0.020**
Manose	LL	1.95 ± 0.16	8.63 ± 1.78	0.91 ± 0.07	1.35 ± 0.17	1.76 ± 0.21
	HL	1.50 ± 0.26	0.91 ±0.26	1.15 ± 0.08	1.42 ± 0.30	1.55 ± 0.28
	P-value	0.195	**0.005**	0.061	0.847	0.568

Data are given as averages ± standard errors. P-values ($P<0.05$) that are statistically significant are presented in bold ($n = 4$).

similar RGR-D under high light (**Figure 2C**). One possible cause for this would be a higher starch content in the Wolffioideae than the Lemnoideae under this condition.

Duckweed Carbohydrates

Figure 3 shows a comparison of the carbohydrate composition for each species of duckweed grown under low (20 μmoles

m^{-2} s^{-1}) and high light intensity (500 μmoles m^{-2} s^{-1}) conditions. Two different statistical approaches were taken. One compares the differences among species (ANOVA One-way) under the two light conditions (Supplementary Table 1), and the other compares effects of light on each species, using a *t*-test for the two light treatments (**Table 1**). With respect to the species studied in this work, light intensity had relatively small effects on carbohydrate partitioning in terms of starch and soluble sugars (**Figure 3,** Supplementary Table 1). On average, accounting only for the carbohydrates, soluble sugars represented 17.1%, starch 6.4%, and cell walls 47.9% of the biomass (**Figure 3,** Supplementary Table 1). Other components such as lipids, proteins, and secondary metabolites accounted for about 28.6% (**Figure 3**). As reported by previous workers, we found a constant low level of 3% lignin in these aquatic plants, irrespective of the light conditions and relative growth rates (data not shown).

Table 1 shows the carbohydrate composition of the five species of duckweeds used in this work. Under high light, sucrose and raffinose increased in most of the species studied, while fructose and glucose decreased in the Lemnoideae and increased in Wolffioideae. An exception was *W. caudata*, for which glucose and raffinose decreased significantly under high light (**Table 1**). Statistical differences could be seen between Lemnoideae and Wolffioideae under high light treatment, with induction of starch accumulation in Wolffioideae (Supplemental Table 1 and **Table 1**). The analysis of cell wall components under the two light treatments revealed the following trends upon increase in light intensity: (1) uronic acids decreased for most species except for *L. punctata,* where the main features were a significantly lower uronic acids and a higher mannose content in low light in comparison with the other species; (2) all the monosaccharides except for mannose and glucose decreased in Lemnoideae species whereas we observed an increase in most of them in Wolffioideae.

Figure 4 and **Table 1** show the trends in apiose in the cell walls for the species studied. There is a significant trend toward higher apiose content in Lemnoideae in comparison with Wolffioideae. Furthermore, under higher light intensity, there was a trend toward a decrease in apiose for Lemnoideae (significant for *L. gibba*) in contrast to the inverse trend toward higher apiose observed with the two Wolffioideae species (more significantly for *W. borealis*) (**Figure 4**). The apiose content evaluated was only structural, found in the pectin fractions.

Principal Component Analysis (**Figure 5**) using all the variables confirmed the observations above and revealed that starch and apiose contents are negatively correlated. In the case of low light, this can be explained by the higher capacity of growth with *S. polyrhiza* (**Figure 5A**), which could be the reason for its clear separation from all the other species tested (PC2). However, under high light (**Figure 5B**), the species from Lemnoideae, which has higher apiose content, propagated faster (RGR-F), whereas the Wolffioideae species grew slower (RGR-F), concomitant with accumulating higher amounts of starch.

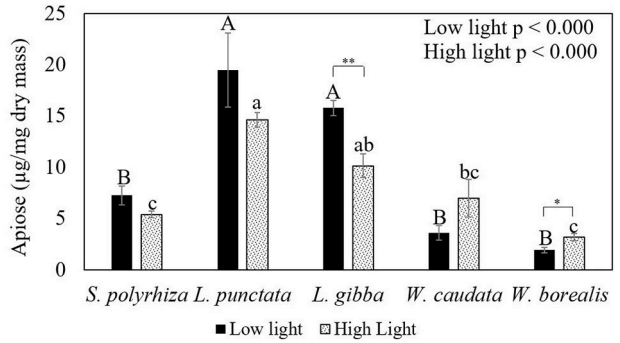

FIGURE 4 | Comparative analysis of the apiose content in the cell walls of duckweeds (*S. polyrhiza, L. punctata, L. gibba, W. caudata*, and *W. borealis*) cultivated under two light intensity (20 and 500 μmol photons m^{-2} s^{-1}) conditions. Data are the averages ± standard errors ($n = 4$). Letters in capital on top of darker bars are the significant differences by Tukey's test ($p < 0.05$) for low light. Lowercase letters on top of gray bars mean significant differences regarding high light. Asterisks indicate significant differences by *t*-test ($p < 0.05$) for species in two light intensities.

DISCUSSION

In this study, we found significant differences between Lemnoideae and Wolffioideae regarding growth, starch content and cell wall components. For the most part, both subfamilies of duckweeds follow the pattern known for most plant species, i.e., that lower starch content is correlated with higher growth rates (Schulze et al., 1991; Sulpice et al., 2009; Zeeman, 2015). However, our study revealed that Lemnoideae apparently differs from Wolffioideae by displaying higher growth rates with relatively low starch contents under high light condition, while *W. borealis* and *W. caudata* (Wolffioideae) display growth limitation under higher light conditions with a concomitant increase in starch accumulation (**Figures 2C,D**). Our results suggest that the increase in biomass of Wolffioideae under high light is not solely due to an increase in the number of fronds, but may be driven largely by the fact that the fronds become heavier due to the accumulation of starch (**Table 1,** Supplementary Table 1). Thus, there seems to be a limitation for growth that is unrelated to light quantity and possibly associated with the internal control of carbon allocation and/or photosynthetic capacity. It is important to note that non-structural carbohydrates may vary considerably in duckweeds growing in the wild. Indeed, Xiao et al. (2013) compiled data and found that starch in duckweeds can vary from 3 to 75%. The authors concluded that this variation is probably due to geographical location and nutritional factors. Despite the fact that variations in the wild can occur, this is not valid for the interpretation of our results, since the species used in this work were cultivated under the same controlled conditions.

Duckweed biomass has been reported to display higher proportions of soluble compounds, which contrasts with the relatively lower proportions of structural carbohydrates (Xu

et al., 2011; Xiao et al., 2013; Yin et al., 2015; Li et al., 2016). However, our study revealed that structural carbohydrates make up almost half of the biomass in all species analyzed (**Figure 3**). The cell wall of duckweeds has been thought to display higher percentages of pectin (Avci et al., 2018). Duff (1965) was the first to report apiose occurrence in duckweed. This five-carbon sugar was found by Hart and Kindel (1970) as a major constituent of apiogalacturonan in duckweeds (*Lemna minor*). More recently, Avci et al. (2018) determined the structure of several pectic polysaccharides (rhamnogalacturonans, apiogalacturonan, and xylogalacturonan) from 12 species of duckweed and found that the ones from the subfamily Wolffioideae contain lower proportions of apiogalacturonan. These authors also highlight that there is a trade-off between apiogalacturonan and xylogalacturonan in duckweeds, with the latter polysaccharide being higher in Wolffioideae. Xylose can be found in xylans, xyloglucan, arabinoxylans, rhamnogalacturonan, and xylogalacturonan (Guyett et al., 2009; Bar-Peled et al., 2012). This sugar is reported to be lower in duckweeds (Ge et al., 2012; Zhao et al., 2014). However, in the present study, we found that xylose represented approximately 22% of the cell wall (82.84 μg mg^{-1} DM), decreasing in Lemnoideae (0.68 times) and increasing in Wolffioideae (1.7 times) with an increase in light intensity during growth (**Table 1**, **Figure 5**). It would be interesting to examine the molecular basis for this variation and the biochemical consequences in terms of cell wall architectural differences between these subfamilies. The recently reported high-quality reference genome for *S. polyrhiza* (Michael et al., 2017) and the soon-to-be completed *Wolffia australiana* genome (E. Lam, unpublished work) may help to shed light on this aspect.

It has been hypothesized that the presence of apiose (i.e., apiogalacturonans) in duckweeds can be related to their expected need to efficiently assimilate boron (Matoh, 1997) due to the known ability of apiose to specifically form complexes with boron in plant cell walls (Fleischer et al., 1998; Matsunaga et al., 2004). While apiose is present in rhamnogalacturonan II of plants, the existence of apiogalacturonan appears to be unique to aquatic plants such as duckweed (Hart and Kindel, 1970; Mohnen, 2008). We thus hypothesize that the presence of significant amounts of apiogalacturonan in duckweed cell walls could be an evolutionary adaptation of this family of aquatic plants to thrive in their habitat. Since aquatic environments usually have a concentration of boron that is 300 times lower than the average in terrestrial environments (Power and Woods, 1997; Shorrocks, 1997; Lemarchand et al., 2000), it would be reasonable to think that the concentration of apiogalacturonan in the cell walls could be an adaptive factor related to their rapid growth on the water surface.

In addition to the potential relationship with boron adsorption, it is also known that reduced cross-links in rhamnogalacturonan II are related to decreased plant growth (Ishii et al., 2001). The recent report that the structure and cross-linking of this polysaccharide in Lemnoideae and Wolffioideae is conserved (Avci et al., 2018) further strengthens the hypothesis for the positive contribution of apiogalacturonans to enhanced duckweed growth.

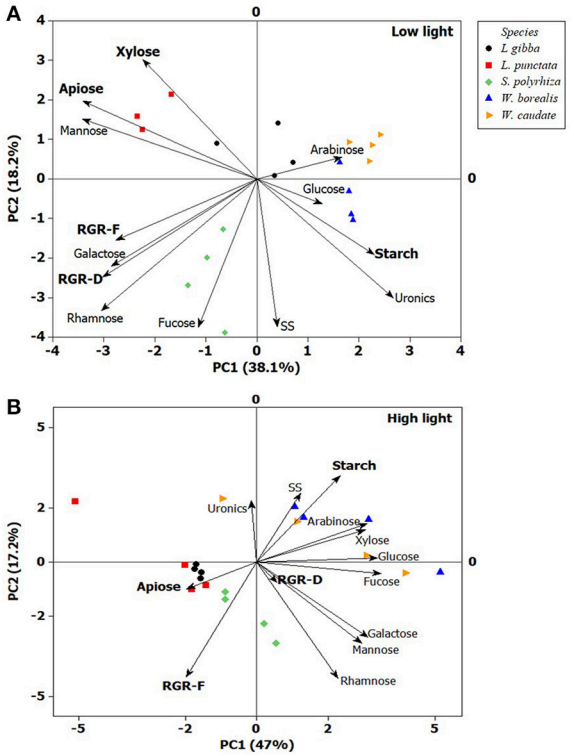

FIGURE 5 | Principal Component Analysis (PC1 and PC2) of duckweeds (*S. polyrhiza, L. punctata, L. gibba, W. caudata*, and *W. borealis*) grown under two light intensities. **(A)** low light (20 μmol photons m^{-2} s^{-1}) and **(B)** high light (500 μmol photons m^{-2} s^{-1}). Xylose, apiose, mannose, arabinose, glucose, galactose, and rhamnose are cell wall-related monosaccharides, uronic acids (uronics) is a component of pectin and some hemicelluloses, starch and SS (Soluble Sugars) represent non-structural carbohydrates, and RGR (Relative Growth Rate) based on number of fronds (RGR-F) and dry mass (RGR-D). The vector values and statistical analyses are in the Supplementary Table 2. ($n = 4$).

In the present study, a trade-off between the accumulation of starch and apiose (probably apiogalacturonnan) has been observed, with higher starch and low apiose levels in Lemnoideae and the reverse trend in Wolffioideae. This was found for 4 out of the 5 species studied. The exception of this was *L. punctata*, in which starch did not vary under different light intensities and uronic acids were rather low under low light when compared to the other evaluated species. A possible explanation for these findings (see Supplementary Figure 3 for some of the possible metabolic pathways present in duckweeds) is that the light intensity used in our experiment was far too low for *L. punctata* in comparison to the other species studied here. This led to a carbon flow that, although still supporting some growth, did not afford cells to establish two of their substantial carbohydrate sinks (starch and pectins). The absence of an increase in starch in the

non-structural carbohydrate pool, at the same time as the comparatively low uronic acids levels in the cell walls of *L. punctata*, is consistent with the hypothesis that *L. punctata* may have rechanneled carbon metabolism toward the GDP-mannose pathway under low light. In fact, this hypothesis is corroborated by the observation of a 10-fold higher accumulation of mannose in this species in comparison with *W. caudata* (**Table 1**).

More apiose was found in the species of Wolffioideae cultivated under high light, with a concomitant increase in their growth rates. Nevertheless, their growth rates in terms of development (RGR-F) were still lower and their starch content higher when compared to those of the three Lemnoideae (except for *L punctata* in this work) species studied under the same conditions. In the Lemnoideae species, where apiose is usually high even when grown under low light intensity, their higher growth rates were consistent with lower accumulation of starch as well.

In sum, our results suggest that apiogalacturonans in duckweeds may be a rate-limiting factor for growth of duckweeds, especially in species belonging to the subfamily of Wolffioideae. If proven true in further screening of additional species and accessions in this subfamily of duckweeds, the levels of apiose and apiogalacturonans may be useful biomarkers for identification of high growth rate duckweed strains for commercial applications.

AUTHOR CONTRIBUTIONS

MB and EL planned the work, DP and EI performed the experiments, DP and AG analyzed the data, MB, AG, DP, and EL wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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Development of Efficient Protocols for Stable and Transient Gene Transformation for *Wolffia Globosa* Using *Agrobacterium*

*P. P. M. Heenatigala[1,2], Jingjing Yang[1], Anthony Bishopp[3], Zuoliang Sun[1], Gaojie Li[1], Sunjeet Kumar[1], Shiqi Hu[1], Zhigang Wu[1], Wei Lin[1], Lunguang Yao[4], Pengfei Duan[4] and Hongwei Hou[1]**

[1] The State Key Laboratory of Freshwater Ecology and Biotechnology, The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Wuhan, China, [2] Inland Aquatic Resources and Aquaculture Division, National Aquatic Resources Research and Development Agency, Colombo, Sri Lanka, [3] Centre for Plant Integrative Biology, University of Nottingham, Nottingham, United Kingdom, [4] Collaborative Innovation Center of Water Security for Water Source Region of Mid-Line of South-to-North Diversion Project, College of Agricultural Engineering, Nanyang Normal University, Nanyang, China

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***Correspondence:**
Hongwei Hou
houhw@ihb.ac.cn

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Members of the *Wolffia* genus are fascinating plants for many biologists as they are the smallest flowering plants on Earth and exhibit a reduced body plan that is of great interest to developmental biologists. There has also been recent interest in the use of these species for bioenergy or biorefining. Molecular and developmental studies have been limited in *Wolffia* species due to the high genome complexity and uncertainties regarding the stable genetic transformation. In this manuscript we present new protocols for both stable and transient genetic transformation for *Wolffia globosa* using *Agrobacterium tumefaciens*. For the transient transformation, we used *Wolffia* fronds whereas we used clusters for the stable transformation. As proof of concept we transformed two synthetic promoter constructs driving expression of the GUS marker gene, that have previously been used to monitor auxin and cytokinin output in a variety of species. Using these approaches we obtained a Transformation Efficiency (TE) of 0.14% for the stable transformation and 21.8% for the transient transformation. The efficiency of these two methods of transformation are sufficient to allow future studies to investigate gene function. This is the first report for successful stable transformation of *W. globosa*.

Keywords: transient transformation, stable transformation, duckweed, *Wolffia globosa*, auxin, cytokinin

INTRODUCTION

The genus *Wolffia* is a member of Lemnaceae or duckweed family. This family comprises five genera, of which members of *Wolffia* and *Wolffiella* are, the smallest angiosperms in the world (Appenroth et al., 2013). *Wolffia* plants consist of a highly reduced structure, comprising a single thallus or frond, less than 1 mm in size (Landolt, 1986; Bernard et al., 1990). *Wolffia* fronds are globular or oval shaped and the upper surface is flattened. Unlike many other members of the

Lemnaceae, *Wolffia* plants are rootless. It has been reported that duckweeds absorb nutrients and water through the underside of their fronds (Leng, 1999) most likely making the root functionally redundant (Hillman, 1961; Anderdon et al., 1973). *Wolffia* increases their biomass mainly through asexual budding by producing daughter fronds within a single side pouch (basal cavity) of the mother frond (Sree et al., 2015; Ziegler et al., 2015). The process of vegetative reproduction allows *Wolffia* plants to produce genetically homogeneous populations when cultivated from a single clone and to show vigorous growth in natural environments. (Bonomo et al., 1997; Xu et al., 2011). Under favorable conditions, *Wolffia* plants are able to double their population size within 30 h (Skillicorn et al., 1993).

Under optimized growth conditions, duckweeds contain high protein with the crude protein content reaching up to 45%. Therefore, there has been increased interest in the use of *Wolffia* as a good protein source particularly for use in animal feed (Skillicorn et al., 1993; Ismail, 1998). As duckweed species have been shown to secrets certain target products into the culture medium (Firsov et al., 2015), they may be able minimize the purification cost of target proteins in duckweed based bioreactors. As many duckweeds including *Wolffia* species reproduce clonally, this allows to be grown in closed system bioreactors, which would minimize the chance of accidental release of transgenic plants (Kruse et al., 2002; Sree et al., 2015). Together with the ability of *Wolffia* species to produce genetically uniform populations from a single clone, these characteristics have made *Wolffia* easy and inexpensive to cultivate in bioreactors (Thompson, 1989). We therefore predict that research interest in duckweed species will increase within the coming years. *Wolffia* species present ideal model systems with which to study for physiological, biochemical, and genetic properties of duckweeds (Anderdon et al., 1973).

One bottleneck preventing greater use of *Wolffia* in commercial applications relates to uncertainty concerning the stable genetic transformation of *Wolffia*. Previously there have been reports of transient transformation for a number of *Wolffia* species including - *W. australiana, W. globosa,* and *W. columbiana* (Boehm et al., 2001; Kruse et al., 2002; Friedrich, 2005; Pham et al., 2010). However protocols for the stable transformation of *Wolffia* have only been reported in *W. arrihiza* (Khvatkov et al., 2015a). Estimations of genome size based on flow cytometry, have shown that *W. arrihiza* has genome size of approximately 1,881 Mbp, over 5-fold larger than the other *Wolffia* species, such as *W. australiana* (Wang et al., 2011). Therefore, there is great need to establish new protocols to allow the stable transformation of other *Wolffia* species. In this paper, we present new protocols for both the transient and stable transformation of *W. globosa.* As proof of concept we introduce to synthetic reporters (*TCS* and *DR5*) driving expression of the *GUS* reporter gene, that have previously been shown to report the cytokinin and auxin signaling output in a variety of species (Benkov et al., 2003; Müller and Sheen, 2008). Although most studies detailing novel methods of transformation use constitutive promoters as this ensures correct identification of all transformed cells, we selected to use the synthetic hormone reporters instead as they have been used successfully in our laboratory in a variety of aquatic plants, including *Hygrophila difformis* (Li et al., 2017) and *Spirodela* and *Lemna* (data unpublished). These transgenic plants will have the advantage that they will provide tools for further studies wishing to investigate growth and development in *Wolffia*, and as such, provide value beyond this study.

MATERIALS AND METHODS

Plant Material and Preparation of Explants

Wolffia globosa (5563) was collected from a native population in Central China (City of Wuhan, Hubei province) at Wuhan Botanical Garden, Chinese Academy of Sciences (CAS) (30.54°N and 114.42°E). Previous work has sampled from this area confirmed the duckweed population as *Wolffia globosa* based on morphological characteristics including absence of pigment cells in the fronds, the number of stomata per frond, the size and shape of the frond and ecological adaptations and confirmed based on chloroplast mat K sequencing (Yuan et al., 2011). *W. globosa* fronds was cultured in SH medium (Schenk and Hildebrandt, 1972) and used as explants in this study. Explants were cultured under sterile conditions at 25 ± 1°C under the white light of 85 μmol $m^{-2}s^{-1}$ 16 h day/8 h night photoperiod.

Cloning of Reporter Constructs

Two vector constructs were used for experiments (**Figure 1**). The cytokinin response element (TCS) and auxin response element (DR5) were synthesized and inserted to the binary vector pKGWFS7.0 (http://www.transgen.com.cn/) using the Gateway technology (Invitrogen). The constructs were mobilized into the commercially available disarmed *Agrobacterium* strains LBA4404 and EHA105 (http://www.transgen.com.cn/) and used for the transformation experiments.

Agrobacterium-Mediated Transient Transformation of *W. globosa*

Preparation of Agrobacterium

A. tumefaciens strains (LBA4404 and EHA105) harboring the plasmids TCS*::GUS*/pKGWFS7.0 and *DR5::GUS*/pKGWFS7.0 were cultivated in the following way. Bacteria from stock cultures were subcultured in 5 ml of Agro LB liquid medium (**Table 1**) supplemented with spectinomycin and rifampicin (BIOSHARP–China) and incubated at 28°C for 48 h with shaking. Cells were harvested by centrifugation at 5,000 rpm (Eppendorff 5804R, USA) for 15 min and re-suspended with 10 ml of infection medium (**Table 1**). Acetosyringone (AS) (Sigma-Aldrich) was only added to the infection medium after autoclaving.

Inoculation and Co-cultivation of W. globosa

Approximately 1 g of explants (**Figure 2A**) were placed in a 2 ml sterilized Eppendorf microcentrifuge tube containing 1 g of sterilized glass beads (1 mm). Tubes were filled with *Agrobacterium* suspended in Infection Medium and shaken at around 180 rpm for 15 min whilst maintaining the temperature at 28°C using an incubator orbital shaker (Crystal, IS-RS D3 - China). One microliter of silwet L-77 was added to the each tube after shaking. A vacuum of approximately 0.8 kg/cm^2 was applied

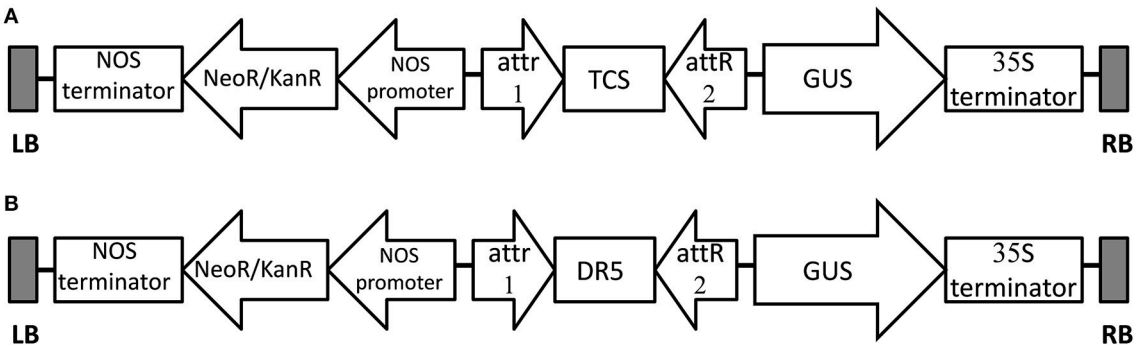

FIGURE 1 | Schematic representation of T-DNA region of the expression vector TCS*::GUS*/ pKGWFS7.0 **(A)** and *DR5::GUS*/ pKGWFS7.0 **(B)**, attR & attR2 - Recombination sites for the Gateway LR reaction.

TABLE 1 | Composition of the media used in transient transformation.

Media type	Composition
Frond Culture Medium	SH + 2% Sucrose + 0.6% Agar
Agro LB medium	Tryptone 10 g l^{-1} + Yeast extract 5 g l^{-1} +NaCl 10 g l^{-1} + Spectinomycin 100 mg l^{-1} + Rifampicin 20 mg l^{-1}, (pH - 7)
Infection Medium	Sucrose 50 g l^{-1} + $Mgcl_2$(1 M) 10 ml l^{-1} + AS 10 μM l^{-1}
Growth Medium	Liquid SH + 1% Sorbitol + 5% Sucrose + AS 10 μM l^{-1}
Selection Medium	Liquid SH + 2% Sucrose + Cefotoxime 300 mg l^{-1} + G418 40 mg l^{-1}
Frond Induction Medium	SH + 2% Sucrose + 0.6% Agar + Cefotoxime 150 mg l^{-1} +G418 40 mg l^{-1}

twice (each time for 15 min and subsequently released quickly). Explants were transferred to sterilized filter papers soaked in Growth Media (**Table 1**) and co-cultivated with the *Agrobacteria* for 48 h.

Selection of Transformants

After 48 h of co-cultivation, explants were transferred into Selection Medium containing G418 and cefotaxime (BIOSHARP–China) to select resistant fronds and eliminate *Agrobacteria* (**Table 1**). After 1 week, resistant fronds were transferred to the Frond Induction medium (**Table 1**) and cultured for another week before the β-Glucuronidase (*GUS*) assay.

To identify suitable concentration for the selection of tranasgenic explants, trials were conducted with different concentrations of G418 (25, 35, 40, 50, 60, and 80 mg l^{-1}) in triplicate.

GUS-Expression Assays

The histochemical assay for *GUS* activity in transgenic explants was performed according to the methodology described by Jefferson et al. (1987). G418 resistant fronds cultured in frond induction medium were immersed in Histochemical Buffer containing 0.5 mg ml^{-1} X-gluc, vacuum infiltrated (0.8 kg/cm^2) for 30 min, and then incubated at 37°C for 12 h. After incubation, explants were washed with deionized water and 40% ethanol prior to observations under the stereomicroscope (Shunyu EX20, China). Transformation efficiency (TE) was calculated as percentage of *GUS* positive explants in the total number of explants.

Agrobacterium-Mediated Stable Transformation of *Wolffia*

Cluster and Callus Induction

Both *Wolffia* calli and clusters (fused aggregates) (**Figures 2B–D**) were used for *Agrobacterium* mediated stable transformation trials. Clusters and calli of *W. globosa* were induced as described by Khvatkov et al. (2015b) using the preconditioned frond explants, and maintained in SH medium. To induce clusters and calli, explants were cultured in Cluster Induction Medium for around 4 months and then transfer to Callus Induction Medium (Khvatkov et al., 2015b).

Transformation

A. tumefaciens (EHA105 containing *TCS::GUS*) was subcultured as 4 lines on solid YEB medium containing 0.8% agar, rifampicin and spectinomycin (**Table 2**) and grown for 48 h at 28°C. After 48 h, 4 lines were scraped and suspended in 8 ml of liquid YEB (without antibiotics). The optical density of the bacteria suspension was 0.7 ± 0.1 at 600 nm (OD_{600}). Subsequently 2 g of *Wolffia* clusters were placed in falcon tube with 5 ml of bacteria suspension and 1 g of sterilized glass beads (1 mm). The tubes were subjected to 180 rpm of vigorous shaking for 30 min at 28°C in an orbital shaker. Then clusters were blot dried and co-cultivated for 72 h on filter papers soaked in Co-cultivation Medium (**Table 2**). Clusters were then cultured on Resting Medium (regeneration and elimination of *Agrobacteria*)

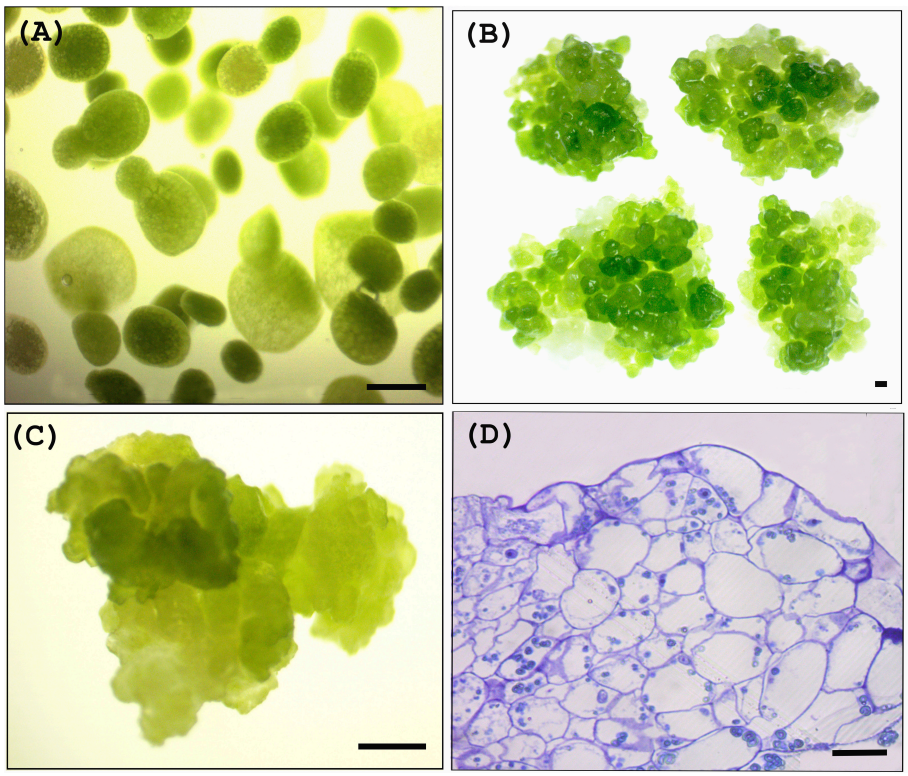

FIGURE 2 | *Wolffia* plant materials used for transient and stable transformation. **(A)** *Wolffia* explants used for transient transformation, **(B)** *Wolffia* clusters used for stable transformation trials, **(C)** *Woiffia* calli used for stable transformation trials, **(D)** Cross section of calli showing cell distribution *bars*, 50 μm **(A,C)** 500 μm **(B,D)**.

TABLE 2 | Composition of the media used in stable transformation.

Media type	Composition
YEB medium	Yeast extract 6 g l^{-1} +Tryptone 5 g l^{-1} + Sucrose 5 g l^{-1} + $MgSO_4.7H_2O$ 0.5 g l^{-1} + Rifampicin 20 mg l^{-1}+ Spectinomycin 100 mg l^{-1} (pH-7)
Co-cultivation Medium	Liquid SH +2% Sucrose +2 mg l^{-1} 2, 4-D + 2 mg l^{-1} 6 BA
Resting Medium	SH + 2% Sucrose +2 mg l^{-1} 2, 4-D + 2 mg l^{-1} 6 BA+ Cefotaxime 300 mg l^{-1}
Selection Medium	SH + 2% Sucrose + 0.6% Agar + Cefotaxime 300 mg l^{-1} + G418 40 mg l^{-1}
Frond Induction Medium	SH + 2% Sucrose + 0.6% Agar + Cefotaxime 150 mg l^{-1}

(**Table 2**) for 2 weeks and transferred to Selection Medium (**Table 2**). Selection was carried out for at least 4 weeks (first 2 weeks with 2 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2 mg l^{-1} N6-benzyladenine (6-BA) and then without growth regulators for the final 2 weeks). The explants were then transferred to Frond Induction Medium and cultured for another 2 weeks.

All steps of both the transient and stable transformation experiments were carried out at 25 ± 1°C under the white light of 85 μmol $m^{-2}s^{-1}$, using a 16 h day/8 h night photoperiod.

Estimation of Transformation Efficiency and Visualization of Reporters

Transformation Efficiency (TE) was measured as described by Khvatkov et al. (2015a). After 1 month of selection, all resistant fronds from each petri dish were considered as a single transgenic population. TE was calculated as percentages of resistant fronds in the total number of explants.

GUS-Expression Assays

Histochemical assay for *GUS* activity in transgenic explants was performed according to the methodology described under the section GUS-Expression Assays.

Genomic Analysis of Transgene Integration

To detect gene integration within the plant genome we firstly used a PCR assay. Total genomic DNA from the putative transgenic and wild-type *Wolffia* explants was extracted using a plant genome extraction kit, Nuclean Plant Gen DNA kit –CW BIO (http://www.cwbiotech.com.cn/). DNA extracts obtained

were used as template to amplify the *TCS* element and *GUS* gene using specific primers. Primers used for TCS element was *TCS*-F: 5′-GGGACAAGTTTGTACAAAAAAGCAGGCTAG CTTTGCTAGCAAAATCTACA-3′ and *TCS*-R: 5′-GGGGAC CACTTTGTACAAAAAGCTGGGTTGTTATATCTCCTTGGA TCGAT-3′. Primers used for GUS gene was *GUS*-F: 5′-TCAACG GGGAAACTCAGCAAGC-3′ and *GUS*-R: 5′-CCTCCCTGC TGCGGTTTTTCA-3′.

Each PCR reaction mixture of 20 μl consisted of 2.0 μl of 10 × buffer, 0.5 μl of 10 mM dNTPs, 1 μl of reverse and forward primers each at 10 μM, 0.2 μl of Taq polymerase, 14.8 μl of deionized water and 60 ng (0.5 μl) of a DNA template. PCR was carried out for TCS element in a thermal cycler (Eppendorff, USA) at annealing temperature of 65°C and at 60°C for the *GUS* gene.

PCR products were separated on a 1.2% agarose gel and visualized under 300 nm wave length of UV. PCR products were sent for sequencing (http://www.tsingke.net) to confirm the correct sequence.

RT- PCR Detection of the Expression of Gene Integrated

Total RNA from wild and transformed *W. globosa* was extracted using the Trizol reagent (Invitrogen). cDNA was synthesized using 2 μg of total RNA using a Primescript RT reagent Kit (Takara). For RT-PCR the same *GUS*-specific primers (used for genomic PCR) and PCR conditions were used. An *Actin* gene was used as the internal control. As the sequence of the *Wolffia* genome is not available yet, a *Wolffia Actin* gene was first amplified using the degenerate primers. *ActF:* 5′-GTGYTK GAYTCTGGTGATGGTGT-3′ and *ActR:* 5′- ACCTTRATCTTC ATGCTGCTSGG- 3′. PCR was carried out for *Actin* gene at annealing temperature of 57°C. The gene amplified was ligated to the pEASY- T1 simple vector system (Transgene) and sequenced by Tsingke company (http://www.tsingke.net). The sequence obtained was subjected to the homology search using BLAST in National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) to correctly identify the gene.

Statistical Analysis

Significance of the TE parameters are shown as Standard Deviation (SD) of the mean made in triplicates and tested by *tukey*-test using SPSS software version 23.

RESULTS

Transient Transformation of *Wolffia globosa*

Both Transient and Stable methods of transformation have proved highly informative for many avenues of research into a myriad of molecular processes. Stable gene transformation remains the most desirable method for the long-term analysis of gene function or the long-term production of specific compounds. However in many instances, transient gene transformation may be a preferable method. In such instances transient transformation can introduce or silence genes in plants and can be used to manufacture desired protein products. Compared with stable transformation, transient transformation is often versatile, quick and efficient. We therefore set out to establish protocols for both methods of transformation in *W. globosa*.

We developed the transient transformation protocol for *W. globosa* using *A. tumefaciens* strains EHA105 harboring either the *TCS::GUS*/pKGWFS7.0 plasmid or the *DR5::GUS*/pKGWFS7.0 plasmid. In order to optimize the transient transformation protocol, we applied a suite of different variations to the protocol. Whilst some variation in conditions was possible, the incorporation of vigorous shaking with glass beads, vacuum infiltration and incorporation of 10 μM l^{-1} AS to both the inoculation and co-cultivation media were absolute requirements successful transformation, and without these processes transformation was unsuccessful. We calculated the TE based upon the percentage of explants with positive GUS activity. Based on this we noted that transformation with *Agrobacterium* strain EHA105 gave a higher TE (21.8%) than when using the LBA4404 strain (**Figure 3C**). We also noted that the percentage of *TCS::GUS* transformed fronds was higher for both *Agrobacterium* strains than the number of *DR5::GUS* transformed fronds (**Figure 3C**).

We found that, the optimal concentration of G418 for selection of transformed lines based on antibiotic resistance was 40.0 mg l^{-1}. G418 resistant explants were clearly identifiable after 2 weeks of selection. Successful transient transformation was confirmed on putative transformants using a *GUS* showing activity of the GUS transgene under control of either the TCS or DR5 promoters (**Figures 3A,B**).

Stable Transformation

To optimize the protocol for stable transformation of *W. globosa*, we altered several trials with different treatments and tested the efficiency of both *Agrobacterium* strains, EHA105 and LBA4404 harboring the *TCS::GUS*/pKGWFS7.0 plasmid. We only observed the stable transformation using the EHA105 strain. Additionally we observed that the clusters needed to be shaken vigorously with glass beads and the *A. tumefaciens* suspension. Trials conducted without this step were unsuccessful. Successful transformation events occurred when the optical density of *A. tumefaciens* suspension was 0.7 ± 0.1 at 600 nm (OD_{600}). We also identified that the optimal co-cultivation period for the successful stable transformation of *W. globosa* with *Agrobacteria* was 3 days. After co-cultivation, subjecting plant materials for a 14 day period in Resting Medium enhanced the efficiency of transformation of the *TCS::GUS* transformed transgene. Alternative resting periods of either 5, 7, or 20 days resulted in unsuccessful transformation. After the resting period, we conducted selection in the presence and absence of growth regulators (2, 4-D and 6-BA). Omitting these growth regulators from the Selection Media resulted in the formation of no GUS positive plants.

After 4 weeks of selection, we tested G418 resistant transgenic explants for incorporation of the transgene. To confirm the incorporation and expression of the TCS::GUS transgenes, G418 resistant populations were initially analyzed for *GUS* activity and this was confirmed subsequently using molecular approaches.

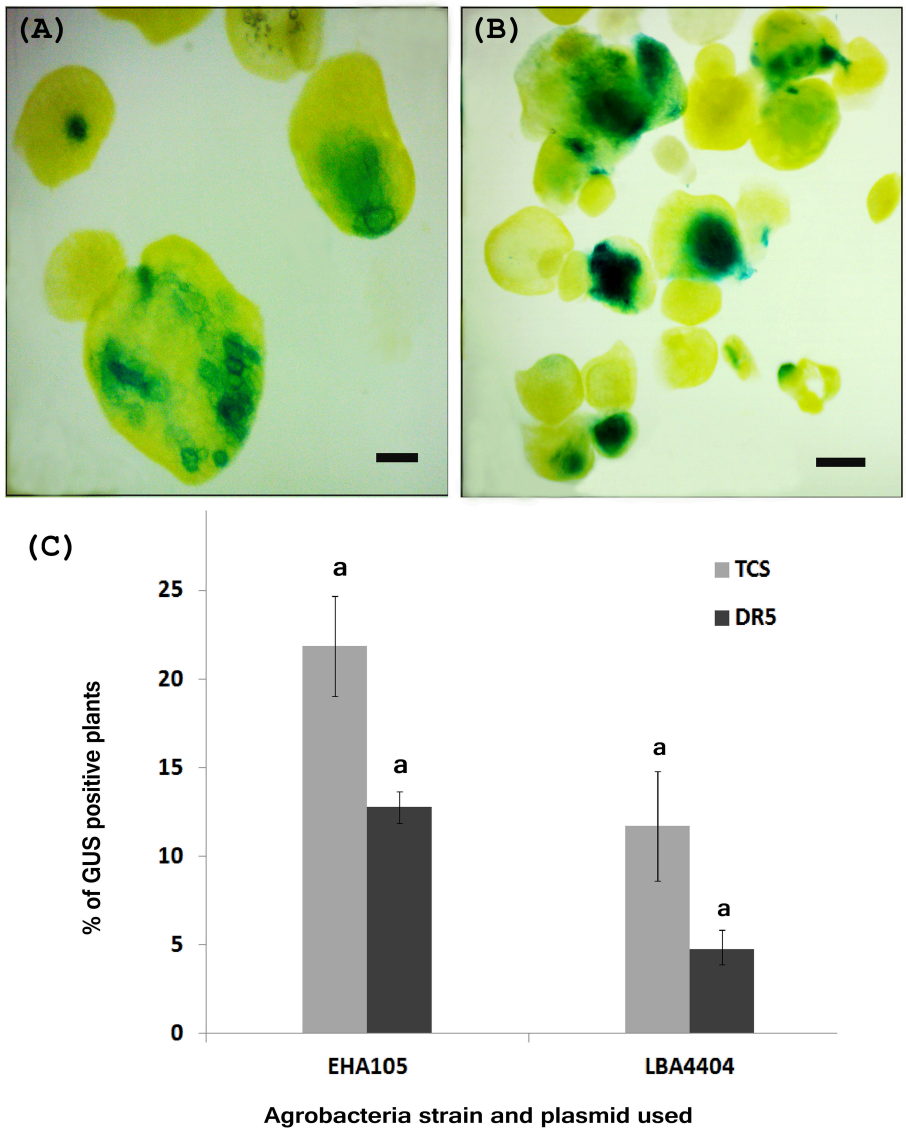

FIGURE 3 | Histochemical *GUS* assay of putative transient transgenic *W. globosa* explants. **(A)** *DR5::GUS* transgenic *Wolffia*, **(B)** *TCS::GUS* transgenic *Wolffia*, **(C)** Percentage of *GUS* positive plants observed during transient transformation, with different *Agrobacteria* strains. Experiments were performed in biological triplicate. Standard deviations are indicated and letters above the bars denote significant difference between the data. *Scale bars* are 500 μm.

GUS staining was present in both mother and daughter fronds of transformed plants but absent from untransformed controls, confirming the stable transformation of the *TCS::GUS* gene (**Figure 4**).

We also verified incorporation of the transgene using a PCR based assay. Following amplification with primers specific for either the TCS promoter element, or the GUS enzyme, we observed expected bands of 404 and 661 bp respectively in transformants (**Figures 5A,B**). No bands were present in our negative controls (wild-type plants). Sequencing of these fragments confirmed that they corresponded to the *TCS* and *GUS* sequences respectively.

In order to test for expression of the GUS gene using RT-PCR, we first had to identify an appropriate internal control. As the *W. globosa* genome has not been sequenced, we performed a homology search for *Actin* gene and designed appropriate *Actin* degenerate primers that showed high similarities with *Actin* gene of other plant species. We used this to identify the sequence of one of the *W. globosa Actin* genes that we could then use in subsequent assays. The sequence identified was 508 bp in length and recorded in Supplementary Data (Sequence - see Supplemental Material).

Following RNA extraction and reverse transcription, PCR products of the anticipated size (661 bp) corresponding to the

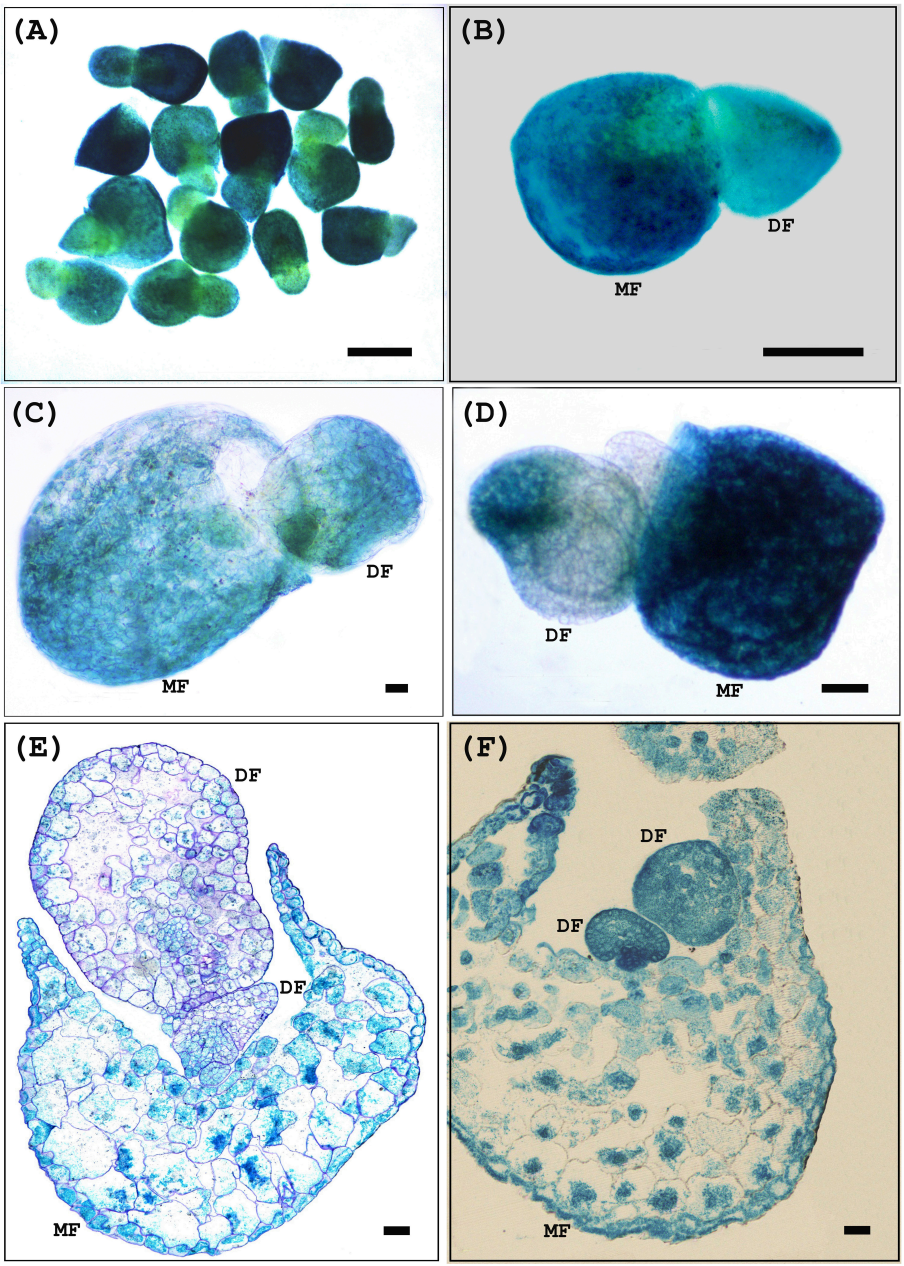

FIGURE 4 | Histochemical *GUS* assay of transgenic *W. globosa* via *Agrobacterium*-mediated stable transformation after the 4 weeks of selection. **(A)** *TCS::GUS* transformed *W. globosa* transgenic fronds **(B)** *TCS::GUS* transformed *W. globosa* transgenic frond showing *GUS* expression in both mother and daughter frond. **(C,D)** *GUS* positive daughter frond comes out from the single side pouch of the stably transformed mother frond. **(E,F)** Cross sections of transgenic *W. globosa* via *Agrobacterium*-mediated stable transformation after histochemical *GUS* assay. *TCS::GUS* transformed *W. globosa* transgenic frond showing *GUS* staining in both mother and daughter frond. MF, Mother Frond; DF, Daughter Frond; Scale bars **(A–D)** - 250 μm, **(E,F)** - 50 μm.

GUS specific primers were obtained with the transgenic *Wolffia* lines but not in the control wild-type explants (**Figure 5C**). Therefore we conclude stable incorporation of the transgene into *W. globosa* using a number of independent assays.

The TE with *TCS::GUS* in stable transformation was 0.14 transgenic plants per 100 explants. However stable transformation with *Wolffia* callus was unsuccessful.

DISCUSSION

Critical Factors for Successful Transient Transformation of *W. globosa*

During transient transformation, we found that addition of AS to the Inoculation and Co-cultivation Medium at the concentration of 10 μM l^{-1} was necessary to achieve high transformation

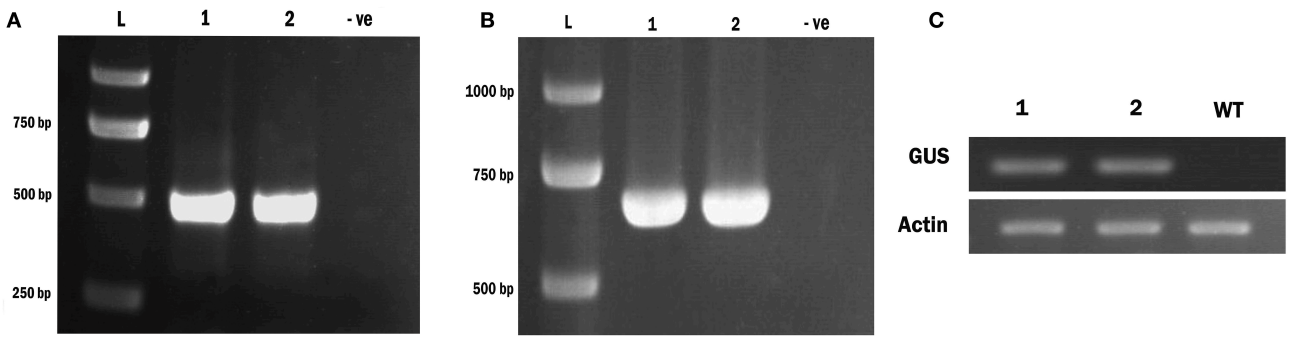

FIGURE 5 | Confirmation of the e *Wolffia* transformed with *TCS::GUS* using PCR & RT- PCR analysis. **(A)** Amplification of *TCS* element from *Wolffia* transformants. **(B)** Amplification of *GUS* gene from *Wolffia* tarnsformants. For both gels a 2,000 bp ladder is used and the sizes of the corresponding bands are indicated. Lanes 1 and 2 contain two independent *TCS::GUS* transgenic plants and lane 3 (marked –ve) containes genomic DNA from a wild plant **(C)** RT-PCR for the GUS enzyme in *Wolffia* transformants. An *Actin* gene was used as internal control. Lanes 1 and 2: putative *Wolffia* transformants, WT, wild-type plant.

efficiency. AS is a phenolic compound that has previously been shown to enhance T-DNA insertion into plants, therefore improving transformation efficiency (Godwin et al., 1991). This needed to be combined with vigorous shaking with glass beads and vacuum infiltration, and presumably these treatments aid the passage of *Agrobacteria* in to the plant cells. Our results are consistent with those of other researchers, for example Boehm et al. (2001), reported unsuccessful transient transformation with *W. columbiana* when either particle or vacuum treatments were not used prior to infection.

We noted that the percentage of *GUS* positive plants was higher in transient transformation conducted using *Agrobacteria* strain EHA105 compared with to using LBA4404 strain (**Figure 3C**) and therefore propose that the *Agrobacteria* strain EHA105 is more suitable for the transformation of *W. globosa*.

Critical Factors for Successful Stable Transformation of *W. globosa*

In this study we found the following factors were critical to obtaining high transformation efficiencies. The concentration of *A. tumefaciens* suspension must have an optical density of 0.7 ± 0.1 at 600 nm (OD_{600}). Other researchers have previously shown that bacterial cell density is an important factor greatly affecting for the TE (Yang et al., 2010; Chhabra et al., 2011). However, there is some variation in what level is optimal, for example, Khvatkov et al. (2015a) reported successful stable transformation of *Wolffia arrhiza* with the cell density of 0.4–0.6 at 600 nm (OD_{600}). Several factors, including plant species, type of explants, *A. tumefaciens* strain vector type and the infection and co-cultivation conditions may collectively affect the gene transformation (Hiei et al., 2000). Therefore, we would expect different outcomes for different *Wolffia* species under different, experimental conditions.

In our stable transformation assays, the *Agrobacterium* strains EHA 105 worked efficiently, and based upon this observation we propose that the EHA105 is more suitable for *W. globosa* transformation than the LBA 4404 strain.

In our experiments we found it necessary that the plant material to be transformed were damaged by shaking with glass beads to improve the accessibility of the infecting bacteria. Several studies have confirmed that wounding plant materials (using either microprojectile bombardment, shaking with glass beads, scratching or slicing) prior to co-cultivation often increased the TE (Grayburn and Vick, 1995; Boehm et al., 2001; Hoshi et al., 2004). Wounding of plant cells can enhance attraction of *Agrobacterium* to the wound site by releasing AS, which also induces the transformation of *A. tumefaciens* virulence genes (Usami et al., 1987). However, Khvatkov et al. (2015a) has reported successful stable transformation of *W. arrhiza* without this treatment and whilst we find that it is needed for *W. globosa*, we note that this may not be the case for other plant species or under different experimental conditions.

Co-cultivation period also a critical factor that affects for the successful transformation and shorter or longer co-cultivation periods lead to unsuccessful transformation (Aileni et al., 2011). In our study, we identified 3 days as the optimal duration for co-cultivation of *W. globosa* and *Agrobacterium*. Longer co-cultivation periods (4–5 days) caused over proliferation of *Agrobacterium* around the plant materials ultimately leading to death of the plant tissues.

After the co–cultivation, plant materials were kept in Resting Medium. This step is essential to all of material to recover from the co-cultivation shock. We found 14 days of resting time before selection to be the optimal conditions for stable transformation in *W. globosa*. This is in accordance with the findings of Khvatkov et al. (2015a). To eliminate *Agrobacteria,* we initially used cefotaxime at a high concentration (300 mg l^{-1}), but we reduced this in subsequent weeks, and eventually found this could be eliminated. Applying cefotaxime in liquid medium was more effective, presumably because the whole surface of the plant was in contact with the antibiotics solution. We therefore found it best to culture plants on filter papers soaked in liquid Resting Medium for the first week after co-cultivation to aid in the successful removal of *Agrobacteria*.

We attempted stable transformation with both clusters and calli of *Wolffia* however we only achieved successful transformation using clusters. This results was similar to the findings of Khvatkov et al. (2015a) and according to their report,

transformation of *Wolffia* with calli was inefficient due to calli undergoing necrosis (Khvatkov et al., 2015a).

CONCLUSIONS

We report here new protocols for both *Agrobacterium* mediated transient and stable genetic transformation systems for *W. globosa*. Using these protocols, transgenic *Wolffia* plants can be produced within 18 days by transient transformation and stable lines can be obtained in 65 days starting with *Wolffia* clusters. The development of these new protocols for transformation open paths to utilize this valuable plant species for a wide range of scientific and commercial processes.

AUTHOR CONTRIBUTIONS

HH planned and designed the research. PH, JY, ZS, GL, SK, SH, ZW, WL, LY, and PD performed experiments and data analysis. PH, AB, HH, SK, and ZW wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted 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/fchem.2018.00227/full#supplementary-material

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Development of *Wolffia arrhiza* as a Producer for Recombinant Human Granulocyte Colony-Stimulating Factor

Pavel Khvatkov[1,2]*, *Alexsey Firsov*[1,3], *Anastasiya Shvedova*[1], *Lyubov Shaloiko*[3], *Oleg Kozlov*[3], *Mariya Chernobrovkina*[1], *Alexander Pushin*[1,2,3], *Irina Tarasenko*[3], *Inna Chaban*[1] and *Sergey Dolgov*[1,2,3]

[1] Laboratory of Plant Gene Engineering, All-Russia Research Institute of Agricultural Biotechnology, Russian Academy of Sciences, Moscow, Russia, [2] Sector of Plant Bioengineering, Nikita Botanical Gardens – National Scientific Centre, Russian Academy of Sciences, Yalta, Russia, [3] Laboratory of Expression Systems and Modification of the Plant Genome "BIOTRON", Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Puschino, Russia

To date, the expression of recombinant proteins in transgenic plants is becoming a powerful alternative to classical expression methods. Special efforts are directed to the development of contained cultivation systems based on cell culture or rhyzosecretion, which reliably prevents the heterologous DNA releasing into the environment. A promising object for the development of such systems is the tiny aquatic plant of *Wolffia arrhiza*, which can be used as a dipped culture in bioreactors. Herein we have expressed the human granulocyte colony-stimulating factor (hG-CSF) in nuclear-transformed *Wolffia*. The nucleotide sequence of hG-CSF was optimized for expression in *Wolffia* and cloned into the vector pCamGCSF downstream of double CaMV 35S promoter. *Wolffia* plants were successfully transformed and 34 independent transgenic lines with hG-CSF gene were obtained, PCR and Southern blot analysis confirmed the transgenic origin of these lines. Western blot analysis revealed accumulation of the target protein in 33 transgenic lines. Quantitative ELISA of protein extracts from these lines showed hG-CSF accumulation up to 35.5 mg/kg of *Wolffia* fresh weight (0.194% of total soluble protein). This relatively high yield holds promise for the development of *Wolffia* -based expression system in strictly controlled format to produce various recombinant proteins.

Keywords: biopharming, hG-CSF, recombinant proteins, transgenic duckweed, transgene expression system, *Wolffia arrhiza*

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United States Department of Agriculture, United States

***Correspondence:**
Pavel Khvatkov
khvatkov1987@gmail.com

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INTRODUCTION

Nowadays a large number of expression systems for recombinant proteins production have been developed. Recombinant proteins are produced in the cells of bacteria, yeast, mammals, and insects (Walsh, 2014; Rader and Langer, 2015; Sysuev and Pokrovskaya, 2015). However, along with the advantages these systems have a number of substantial drawbacks, in particular post-translation modifications and correct folding of many eukaryotic proteins do not occur in bacterial and yeast cells (Jensen, 2006; Merlin et al., 2014; Tschofen et al., 2016). Animal cells are devoid of such shortcomings but their use as bioproducers is limited by reason of recombinant proteins high production cost (Demain and Vaishnav, 2009). Thus, the development of expression systems

combining the advantages of microbial systems (high expression level, relatively low production cost) and systems based on animal cell culture (the complete identity of recombinant protein and its properties to native counterpart) is still urgent.

Apparently the advantages of microbial and animal cell culture expression systems are combined in plant-based platforms to the greatest extent. Basically the protein glycosilation and folding in the higher plants are similar to those in mammalian cells (Strasser, 2016). Unlike animals, plant cells do not contain viruses and microorganisms pathogenic for humans, that substantially simplifies the purification of recombinant proteins for medical purposes (Daniell et al., 2001). Plant cultivation does not require expensive equipment, wherein the agricultural scale of the production guarantees the availability of recombinant proteins in the quantities quite enough for wide therapeutical use (Kaufman, 2011; Wilken and Nikolov, 2012).

The most common current expression systems are based on well-studied agricultural plants, such as tobacco, rice, corn etc. The main problem of these systems is the probability of uncontrolled transfer of heterologous DNA into the environment by seeds, pollen or plant residues during the cultivation of transgenic plant in the field or greenhouse. The fear of this greatly complicates the commercialization of plant-based expression systems. Considerable effort, therefore, has been focused on the development of contained cultivation systems, such as axenic culture of suspension cells or hairy roots (Georgiev et al., 2012; Santos et al., 2016). The contained plant systems can be cultivated in bioreactors, entirely preventing the accidental release of genetically modified plant material to the environment.

Another approach to the development of contained cultivation systems is the using of small aquatic plants—duckweed [*Lemna minor* (L.) and *Wolffia arrhiza* (L.) Horkel ex Wimm.] as recombinant proteins producers. Duckweed are small monocotyledonous plants with a high content of total protein (up to 45% of DW), demonstrating high growth rate (doubling of biomass in 1–6 days) and almost exclusively vegetative reproduction (Armstrong and Thorne, 1984). These plants are capable to photosynthesis *in vitro* and can be effectively and inexpensively cultivated in bioreactors of various types (Gasdaska et al., 2003; Khvatkov et al., 2013).

In *Lemnaceae* family, *Wolffia arrhiza* is the most evolutionary advanced secondary primitively organized species (Wolff, 1992). Plant bodies are tiny; generally globoid to ovoid-ellipsoid or cylindrical; 0.4–1.3 mm long and 0.2–1.0 mm wide; floating on or partially below water surface (Wayne and Thorne, 1984). The most important *Wolffia*'s feature is, that unlike other *Lemnoideae*, it does not have a root system, this property allows the cultivating of *Wolffia* in bioreactors using submergence, which in turn could considerably increase the profitability of recombinant proteins production.

To date, there are already examples of successful expression of commercially important proteins in duckweed plants: the industrial enzyme endoglucanase E1 from *Acidothermus cellulolyticus* (Sun et al., 2007), anti-CD20, anti-CD30, and anti-interferon a2b monoclonal antibodies (Cox et al., 2006; Biolex Therapeutics website), and the protective antigens of influenza virus - peptide M2e (Firsov et al., 2015, 2018), and hemagglutinin (Bertran et al., 2015). Despite that *Wolffia* is very promising plant for biotechnology, yet there is no reports of its use for recombinant proteins expression. The main problem of using *Wolffia* for recombinant proteins expression is the absence of a method for its genetic transformation. The development of such a method allows to start the studies on the development of *Wolffia*—based expression system.

The aim of this study is to explore the feasibility of recombinant proteins expression in nuclear-transformed *Wolffia arrhiza* plants by the example of human granulocyte colony-stimulating factor (hG-CSF). hG-CSF consists of 207 amino acid residues, including a signal peptide (aa 1–29). Mature hG-CSF has a length of 178 aa, it is stabilized by 2 disulfide bonds and glycosylated at position 166. At the present time recombinant hGCSF is widely used for management of various etiology neutropenias in the therapy of patients with HIV, aplastic anemia, pneumonia, leukemia, and others diseases (Harousseau et al., 2000; Babalola et al., 2004; Dale, 2010).

Here, we report the successful Agrobacterial transformation of *Wolffia* and expression of human granulocyte colony-stimulating factor in transgenic plants. Recombinant GCSF accumulated at a high level, sufficient for further studies to create *Wolffia* -based expression system to produce the offered and other recombinant proteins.

MATERIALS AND METHODS

Gene Synthesis and Plasmid Construction

The amino acid sequence of hG-CSF corresponding to the drug Filgrastim (DrugBank: DB00099) was selected for expression in *Wolffia*. The N-terminal signal peptide hG-CSF has been replaced by a corresponding peptide of rice α-amilase (GenBank: AAA33897.1). To optimize the codon composition a table of codon usage in *Lemna gibba* was used (http://www.kazusa.or.jp/codon). The optimization of *hG-CSF* nucleotide sequence was performed by Gene Composer software (Lorimer et al., 2009). The set of overlapping oligonucleotides was designed using Gene2Oligo (Rouillard et al., 2004).

Nucleotide sequence of hG-CSF was synthesized by PCR (Rouillard et al., 2004) and cloned into sites HindIII and EcoRI of plasmid pUC18 followed by sequencing. Then, hG-CSF (in Appendix) sequence was cut out from pUC18 and, using the same sites, cloned into plant expression vector pCamPPVcp, replacing the plum pox virus coat protein gene (Dolgov et al., 2010). The resulting plasmid pCamGCSF was transferred into *A. tumefaciens* strain EHA105 (Hood et al., 1993) and used for *Wolffia* transformation.

Tissue Culture and *Agrobacterium*-Mediated Transformation of *W. arrhiza*

For research, we obtained a population of *Wolffia arrhiza* (L.) Horkel ex Wimm from the collection of Main Botanic Garden of the Russian Academy of Sciences (RDSC Clone *Wolffia* 5564; Supplementary Table 1) (**Figure 1**).

An aseptic population of *W. arrhiza* plants was cultivated on SH medium (Schenk and Hildebrandt, 1972) supplemented with 2% (w/v) sucrose and 0.7% (w/v) agar (Panreac, Spain). 10 plants were placed on each Petri dish filled with culture medium. The cluster structures formed after 4 months of pre-cultivation on MS2G media containing 5.0 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1} N^6-Benzyladenine (BA) (Khvatkov et al., 2015b), were used for transformation. The explants developed at light intensity of 65 μmol m^{-2} s^{-1}, day photoperiod 16 h and temperature 21 ± 1°C.

W. arrhiza was transformed as described by Khvatkov et al. (2015a). Explants were co-cultivated with *A. tumefaciens* containing the plasmid pCamGCSF, with subsequent transfer to SH medium containing 2.0 mg l^{-1} 2,4-D, 2.0 mg l^{-1} BA, 5.0 mg l^{-1} hygromycin B (Hyg) and 100 mg l^{-1} timentin. After2 weeks the explants were transferred to SH medium lack of growth regulators for regeneration. Each newly emerged Hyg-resistant plant was placed into a separate culture tube with W3M media (Dolgov et al., 2013) supplemented with 5.0 mg l^{-1} Hyg for further development.

The inspection of the selected plant material for their regenerative capacity [viz. the development of meristematic tissue (**Figure 3a**) and formation of plant regenerants (**Figure 3b**)] was performed by light microscopy. Preparation of samples and microscopic examination of plant material was carried out according to the procedure described in Miroshnichenko et al. (2017).

Selection of pure transgenic lines was carried out as described by Khvatkov et al. (2015a). Transformation efficiency was determined by dividing the number of explants which formed transgenic populations upon the total number of explants in each experiment (percentagewise).

PCR and Southern Blot Analysis of Transformants

The *Wolffia* genomic DNA was isolated from transformed and non-transformed plants according to the method of Dellaporta et al. (1983). The respective forward and reverse primer sequences for *hpt*II were hptf: 5′-acattgttggagccgaaatc-3′ and hptr: 5′-gacattggggagtttagcga-3′; those for *hG-CSF* were G1for: 5′-gtccaagcttatggcgaagaggatcgcc-3′ and G2rev: 5′-atgaattctcacggttgggcgagatg-3′; those for *virC* were virC1: 5′-gcactatctacctaccgctacgtcatc-3′ and virC2: 5′-gttgtcgatcgggactgtaaatgtg-3′.

For Southern blot analysis, genomic DNA from transformed and non-transfomed *Wolffia* plants in amount of 50 μg was digested overnight at 37°C with 100 U HindIII which cuts the T-DNA of pCamGCSF at a single position. After agarose gel electrophoresis (0.8%), the products of digestion were transferred and immobilized onto Hybond+ membrane (Amersham, USA) according to the manufacturer's instructions. The DNA probe was constructed by PCR using plasmid pCamGCSF as the template, and primers G1for and G1rev. Probe DNA (600 bp) was labeled with alkaline phosphatase using the AlkPhos Direct Labeling Kit (Amersham Bioscience, USA). Prehybridization,

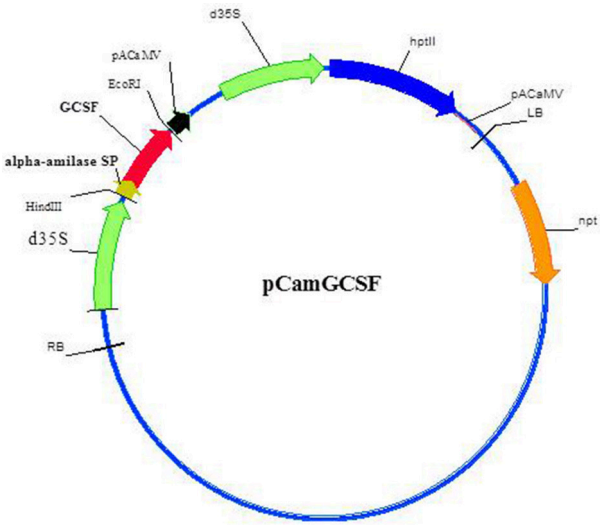

FIGURE 2 | Schematic presentation of pCamGCSF plasmids used for *Wolffia* transformation. d35S, double 35S RNA Cauliflower Mosaic Virus promoter; *GCSF*, human granulocyte colony-stimulating factor coding sequence; *alpha-amilase SP*, nucleotide sequence of rice alpha-amylase signal peptide; *hpt*II, hygromycin phosphotransferase (HPT) coding sequence; pACaMV, 35S RNA Cauliflower Mosaic Virus terminator with polyadenylation signal; RB, right border; LB, left border; *npt*, neomycin phosphotransferase (NPT) coding sequence allowing for *A. tumefaciens* selection.

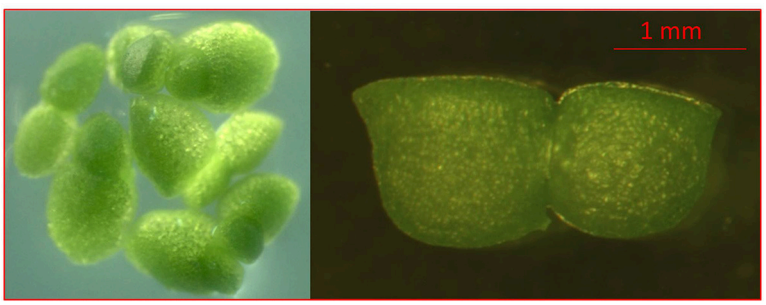

FIGURE 1 | Images of characteristic morphological features of *Wolffia arrhiza*. Dorsal view showing several flat-topped, dark green plants and side view of *W. arrhiza* budding plant showing flattened, dorsal surface of daughter plant.

hybridization (overnight at 60°C) with alkaline phosphatase-labeled probe, and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling Kit protocol. Detection was performed using CDP-Star detection reagent according to the manufacturer's instructions (Amersham Bioscience).

Western Blot Analysis

Transgenic and non-transformed *Wolffia* plants (in an amount of 1 g) were grinded in liquid nitrogen and resuspended in 3x volume extraction buffer (50 mM Tris-HCl (pH7.6), 150 mM NaCl, 5 mM EDTA, 5 mM β-merkaptoethanol, 0.62 μM aprotinin, 8.4 μM leupeptin). Extraction was performed during 40 min at 4°C followed by centrifugation for 20 min at 16,000 g; supernatant was collected for subsequent analysis. Protein concentration in the samples was determined by Bradford method (Bradford, 1976).

Protein electrophoresis (70 μg/lane) was carried out in 10–25% gradient SDS-PAGE. The separated proteins were transfered onto a nitrocellulose membrane (BioRad, USA). Rabbit polyclonal antibody against hG-CSF (diluted 1:1,000; Abcam, UK) and anti-rabbit IgG conjugated with alkaline phosphatase (1:3,000; Pierce, USA) were used. Recombinant hG-CSF (Abcam, UK) served as the positive control. The blots were visualized using chromogenic substrate BCIP/NBT (Fermentas, Lithuania).

ELISA Quantification of hG-CSF Accumulation

The total protein was extracted as described above. The protein samples were serially diluted in PBS and loaded into 96-well ELISA plate (0.25, 0.5, 1.0, and 2.0 μg of TSP per well), using recombinant hG-CSF (Abcam, UK) as the reference standard.

The plates were incubated for 2 h at RT, then washed three times with PBST (PBS with 0.1% Tween 20) for 5 min each and blocked in PBST containing 2% bovine serum albumin (1 h at RT). Hybridization with the primary antibody was performed overnight at 4°C. After washing, the secondary antibody was added and plates were incubated for 1 h at RT followed by the washing. Rabbit polyclonal antibody against hG-CSF (Abcam, UK) was diluted at 1:1,000, anti-rabbit IgG conjugated with alkaline phosphatase (Pierce, USA)—at 1:2,000. The plates were developed for 30 min at RT using TMB Peroxidase EIA substrate (BioRad). The plates were read at 405 nm and the amount of plant—expressed hG-CSF was estimated based on reference standards.

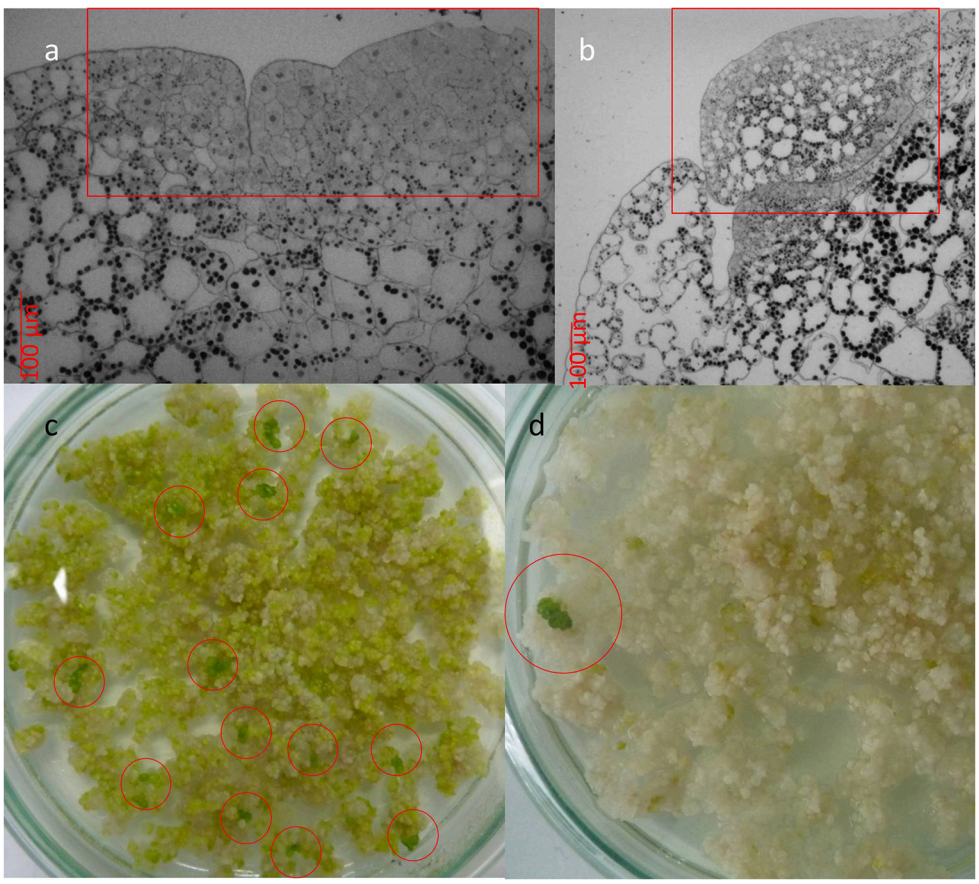

FIGURE 3 | Development of transgenic *Wolffia* plants in the presence of 5.0 mg l^{-1} Hyg in the culture media. **(a)** Meristematic area (squared in red) on the cluster surface which developed after 6 weeks exposure to selective media. **(b)** Globular structures (squared in red) on the cluster surface which developed after 8–10 weeks exposure to selective media. **(c, d)** Multiple **(c)** or single **(d)** formation of whole plants (circled in red) in the presence of 5.0 mg l^{-1} Hyg in the culture media.

Statistical Analysis

The significance of differences in hG-CSF accumulation between *Wolffia* transgenic lines were analyzed by analysis of variance (ANOVA) followed by multiple comparisons of individual averages and evaluation by Duncan's test (Statistica 6.1 software; StatSoft Inc).

RESULTS

Vector Construction and *Agrobacterium*-Mediated Transformation of *Wolffia arrhiza*

We used the data on codon usage in *Lemna gibba*, which belongs to the same *Lemnaceae* family, as *W. arrhiza,* to optimize the codon composition of *hG-CSF* nucleic acid sequence. We assumed that the codon usage do not differ significantly in these two species. Nucleic sequence of *hG-CSF* with the codon composition optimized for the expression in *Lemnaceae* was synthesized and cloned under the control of the CaMV double 35S promoter. The resulting plasmid pCamGCSF (**Figure 2**) was used for hG-CSF expression in *Wolffia* plants, following its *Agrobacterium*-mediated transformation.

In experiments on *Agrobacterium*-mediated transformation, transgenic tissue is distinguishable after 1.5 months under selection on SH medium in the presence of 5.0 mg l^{-1} Hyg. Green meristematic area appeared on cluster surface after 6 weeks of cultivation on SH medium lack of growth regulators (**Figure 3a**, squared in red). Emerald green globular structures were originally developed from meristematic area (**Figure 3a**, squared in red) followed by the arising of the whole plants (**Figures 3c,d**, circled in red). Regeneration of various cultures on the hormone-free medium is a rare but fairly well-known phenomenon. Thus, in addition to the *Wolffia*, the ability to regenerate on hormone-free media is capable, for example, of carrots (Górecka et al., 2009), lotus (Rybczynski and Badzian, 1987), flax (Aycan et al., 2014), parsley (Masuda et al., 2006), beets (Doley and Saunders, 1989), and etc. Obviously, hormone enriched media during the cultivation of explants of these cultures *in vitro* are required solely to maintain them in the state of the callus structures, and not for regeneration.

The morphological differences between Hyg-resistant *Wolffia* plants and non-transformed ones were not observed. In liquid culture the development and growth rate of transgenic plants did not differ from the corresponding characteristics of the non-transformed control plants. In experiments on *Agrobacterium*-mediated transformation of *Wolffia*, 8100 explants were used on the whole; the transformation frequency was 0.42%. In total, 34 independent *Wolffia* lines which developed without signs of hygromycin toxic effect were obtained; these lines were used for further analysis.

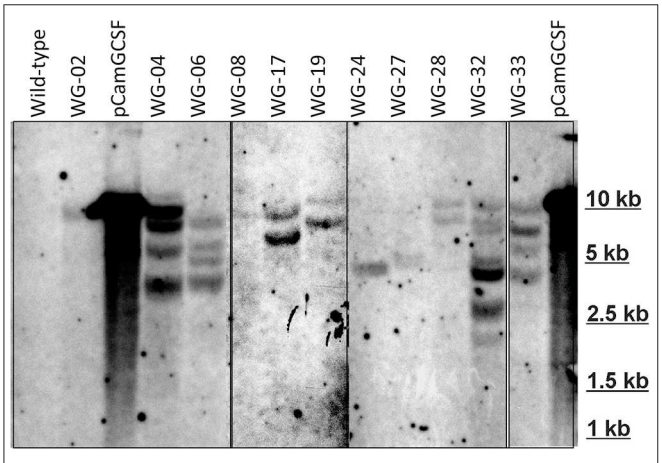

FIGURE 4 | Southern hybridization analysis of some EHA105/pCamGCSF-transformed *Wolffia* plants. DNA (10 μg) digested with HindIII and hybridized with a 600-bp *hG-CSF* probe. pCamGCSF, pDNA (pCamGCSF/HindIII); WG, DNA of transgenic *Wolffia* lines digested with HindIII; Wild-type, genomic DNA of wild-type *Wolffia* plants digested with HindIII.

PCR and Southern Blot Analysis of Transformants

The fragments of the expected size were amplified from the DNA of all analyzed putatively transgenic *Wolffia* lines. As it was shown by PCR using virC1 and virC2 primers, all lines were free of agrobacterial contamination (data not shown). Thus, according to PCR analysis, all of 34 Hyg-resistant *Wolffia* lines contained selective *hpt*II gene and target hG-CSF sequence.

The selected transgenic lines with different hG-CSF expression were analyzed by Southern blot. The obtained results confirmed integration of hG-CSF gene into *Wolffia* genome (**Figure 4**). Based on the hybridization profile, there were one (lines 2, 8, 24, and 27), two (lines 17, 19, 28), and four or more insertions (lines 4, 6, 32, and 33) of the transgene into the obtained lines, which is typical for *Agrobacterium*-mediated transformation. The DNA from non-transformed plants failed to hybridize to the probe.

Analysis of hG-CSF Expression in Transgenic *Wolffia* Plants

Western blot analysis using anti-hG-CSF antibody has revealed the presence of a 20-kDa band corresponding to the recombinant hG-CSF in 33 transgenic lines out of 34 examined (**Figure 5**). hG-CSF from the transgenic plants migrated at the same rate as the control protein. This result confirms the cleavage of the rice α-amylase N- terminal signal peptide, i.e., the correct processing of pre-hG-CSF in *Wolffia* plants. Immunoreactive bands of similar weight were not observed in the protein samples from non-transformed control plants. It should be noted, that there was no noticeable degradation of the recombinant hG-CSF in transgenic plants. In some lines (№ 3, 5, 7, 8, 11, 14, 16, and 19), the target protein was detected at low level; the immunoreactive bands were weak (**Figure 5**). The maximum hG-CSF accumulation was observed in lines WG-04 and WG-32, in others lines the accumulation of the target protein was lower.

According to ELISA, 33 out of 34 transgenic lines expressed hG-CSF in plant tissues (**Table 1**). Quantification of the target protein in transgenic *Wolffia* plants using anti-hG-CSF antibody showed accumulation from 0.36 to 35.5 μg of hG-CSF per 1 g

fresh weight (FW) of *Wolffia*, corresponding to 0.002–0.194% of total soluble protein (TSP). The highest levels of hG-CSF accumulation were observed in lines WG-04 and WG-32 (33.9 and 35.5 $\mu g\ g^{-1}$ FW; 0.191 and 0.194% of TSP, respectively), and the lowest in lines WG-09, WG-13 and WG-14 (0.36, 0.53 and 0.36 $\mu g\ g^{-1}$ FW; 0.002, 0.003, and 0.002% of TSP, respectively) (**Figure 6**).

As a result of our study, our research team was the first to receive transgenic *Wolffia* plants expressing the recombinant human G-CSF. In two lines—WG-04 and WG-32—the target protein was accumulated at a high level—approximately 35 mg of recombinant protein per 1 kg of *Wolffia* FW. Thus, by the example of hG-CSF we have demonstrated the potential of *Wolffia* as a producer of recombinant proteins.

DISCUSSION

We have obtained 34 different lines of *Wolffia* with confirmed transgenic status; the target protein hG-CSF was detected in 33 lines (**Table 1**). Morphologically transgenic *Wolffia* plants did not differ from their non-transformed counterparts. Expression of the foreign protein did not affect the rate of reproduction in liquid culture or plant TSP content. According to ELISA accumulation of recombinant hG-CSF ranged from 0.36 to 35.5 $\mu g\ g^{-1}$ FW of *Wolffia* (0.002–0.2% of TSP).

In our study, we identified almost 100-fold variation in target protein accumulation among the transgenic lines. Variations in recombinant protein expression between independently derived transgenic lines are common (Richter et al., 2000; Sharma and Sharma, 2009). They are often related to differences in the number of transgene copies or in the position within the genome into which the foreign DNA was integrated (Dolgova et al., 2015). Both of these factors might be applied to the transgenic *W. arrhiza* lines that were obtained (**Figure 6**). The Southern blot analysis showed notable differences between the studied lines both as in the number of transgene inserts and in the sites of their integration into the plant genome.

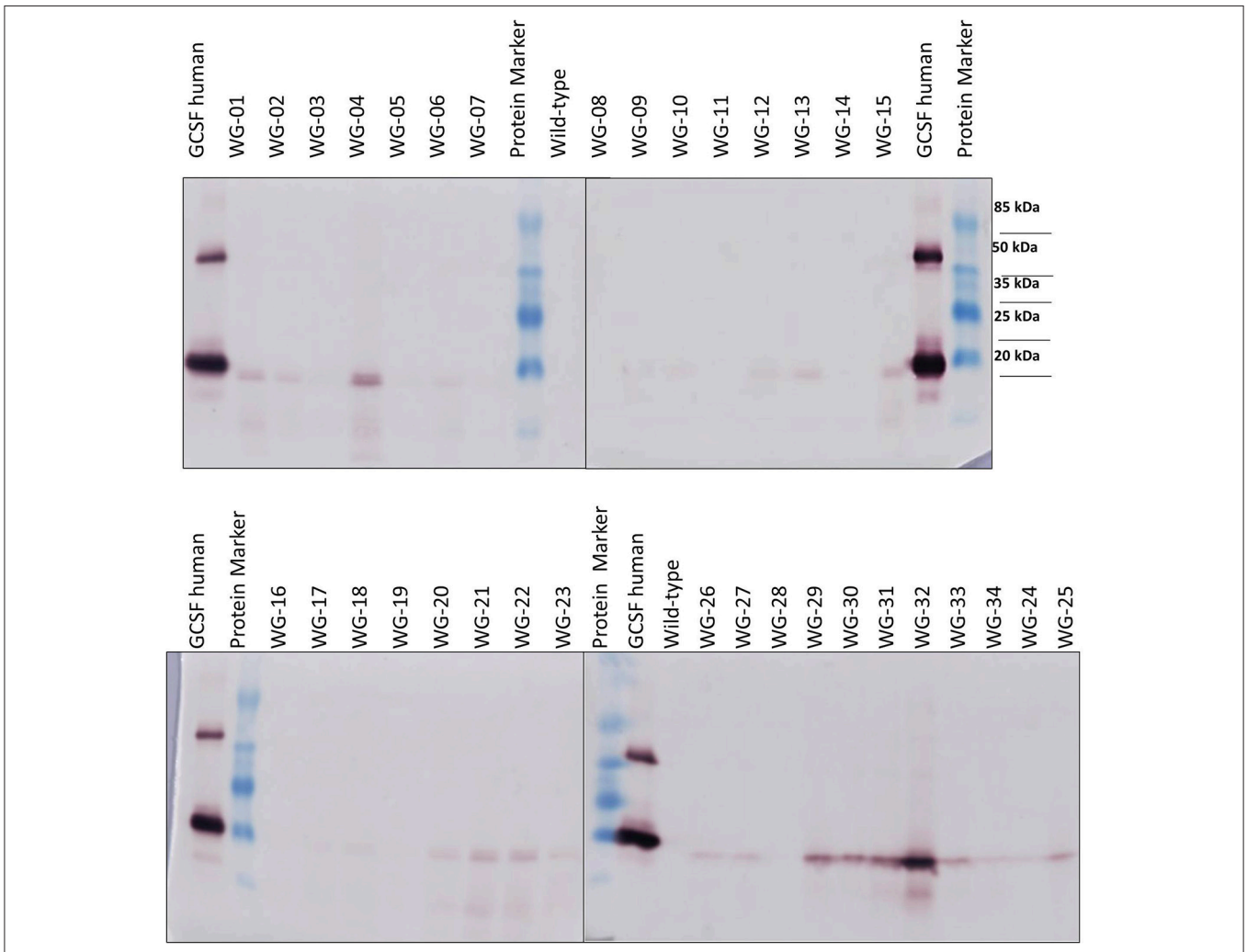

FIGURE 5 | Western blot analysis of hG-CSF expression in some transgenic *Wolffia* lines. Protein Marker, molecular size marker TS 26612 Protein MW Marker; GCSF human, recombinant human G-CSF (200 ng; AbCam, UK).

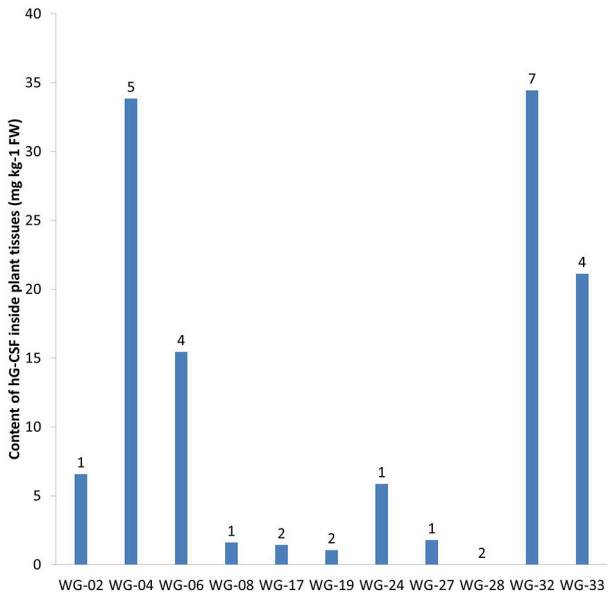

FIGURE 6 | The result of ELISA of 11 transgenic *Wolffia* lines containing *hG-CSF* gene. Numbers designate the number of *hG-CSF* gene insertions into transgenic line genome.

TABLE 1 | Quantitative indicators of hG-CSF expressed in *W.arrhiza* transgenic plants.

Name of transgenic line	Saturation with hG-CSF inside plant tissues	
	Target protein per TSP (%)	mg kg^{-1} of FW
WG-01	0.005 ± 0.002	0.95 a
WG-02	0.037 ± 0.017	6.57ab
WG-03	0.014 ± 0.006	2.49ab
WG-04	0.191 ± 0.052	33.85de
WG-05	0.011 ± 0.006	1.95 a
WG-06	0.087 ± 0.070	15.45bc
WG-07	0.008 ± 0.004	1.42 a
WG-08	0.009 ± 0.001	1.60 a
WG-09	0.002 ± 0.001	0.36 a
WG-10	0.004 ± 0.003	0.71 a
WG-11	0.007 ± 0.002	1.24 a
WG-12	0.004 ± 0.003	0.71 a
WG-13	0.003 ± 0.001	0.53 a
WG-14	0.002 ± 0.001	0.36 a
WG-15	0.007 ± 0.004	1.24 a
WG-16	0.008 ± 0.002	1.42 a
WG-17	0.008 ± 0.004	1.42 a
WG-18	0.005 ± 0.002	0.89 a
WG-19	0.006 ± 0.001	1.06 a
WG-20	0.005 ± 0.003	0.89 a
WG-21	0.005 ± 0.002	0.89 a
WG-22	0.006 ± 0.001	1.07 a
WG-23	0.006 ± 0.004	1.0 a
WG-24	0.033 ± 0.021	5.86ab
WG-25	0.008 ± 0.001	1.42 a
WG-26	0.005 ± 0.003	0.89 a
WG-27	0.010 ± 0.001	1.78 a
WG-28	0.000 ± 0.000	0.00 a
WG-29	0.010 ± 0.004	1.78 a
WG-30	0.028 ± 0.008	4.97ab
WG-31	0.006 ± 0.004	1.07 a
WG-32	0.194 ± 0.060	35.45e
WG-33	0.119 ± 0.023	15.13 c
WG-34	0.004 ± 0.003	0.71 a

Different letters in a column indicate significant differences in variant data according by Duncan's test.

Currently the recombinant hG-CSF used in medicine is produced either in *E. coli* expression system (Filgrastim medication; Vacchelli et al., 2013) or in CHO cell culture (Lenograstim; Serova et al., 2012). The recombinant GCSF manufactured in the bacterial system is non-glycosylated; GCSF produced in CHO cells is glycosylated and does not differ from G-CSF naturally made in humans. Both forms of recombinant hG-CSF are almost similar in pharmacological properties, although there are reports that the glycosylated form has a higher efficiency of mobilizing stem cells than Filgrastim and is more effective at lower doses (Ria et al., 2010; Sari et al., 2013). At that, the cost of GCSF production in CHO culture is significantly higher than in *E. coli* system. Therefore, efforts have been applied to develop alternative expression systems for recombinant GCSF production, including plant- based.

Recombinant GCSF was expressed in plastids of lettuce (Sharifi et al., 2013) and in nuclear-transformed tobacco plants (Sharifi et al., 2012). The level of GCSF accumulation in these studies is not reported. The hG-CSF was also successfully expressed in suspension - cultured tobacco and rice cells. In tobacco BY-2 cells, recombinant hG-CSF was expressed at 17.8 mg/l, the hG-CSF accumulation has been determined in the tissues of initial transgenic callus lines (Nair et al., 2014). Hong et al. (2006) produced hG-CSF in suspension culture of transgenic rice cells (*Oryza sativa* cv. Dong-jin). The expression of recombinant hG-CSF in callus lines amounted up to 185 μg g^{-1} of callus FW. The recombinant GCSF was able to secrete into the culture medium, its accumulation reached 2.5 mg l^{-1} of medium. Recombinant hG-CSF obtained in both studies retained specific biological activity, as shown in cell assays.

In our study, the accumulation of hG-CSF in the most productive lines WG-04 and WG-32 reaches 0.2% of TSP. Such a level of recombinant protein expression (a few of tenths of a percent) is common for nuclear-transformed plants (Lico et al., 2012; Shahid and Daniell, 2016). However, due to the high content of total protein in *Wolffia*, the yield of hG-CSF amounted up to 35 mg kg^{-1} of fresh weight, and this is a fairly good level. It should be mentioned that the cultivation conditions of transgenic *Wolffia* plants have not yet been fully optimized, so under the optimal conditions the hG-CSF yield could be significantly increased. In addition, it is of great interest to study the *Wolffia* recombinant proteins secretion into the culture medium on the example of GCSF; hence this will be the aim of our further research.

In our experiments we have expressed the human G-CSF in nuclear-transformed *Wolffia* plants without impact on plant morphology or growth rate. The accumulation of target hG-CSF protein in the best transgenic line reached 35 mg kg^{-1} FW. In conclusion the present study distinctly demonstrates the feasibility of recombinant proteins expression in nuclear-transformed *Wolffia* plants and opens the way for development of *Wolffia* -based expression systems.

AUTHOR CONTRIBUTIONS

PK, AS, and MC: Obtaining transgenic lines of wolffia; AF, LS, and OK: Western blot analysis and ELISA of transgenic plants; AP Southern blot analysis; IT: Gene synthesis and plasmid construction; IC: Light microscopy; SD: Scientific adviser of this project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00304/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX

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aggggcgatgcccgcgttcgcctccgcctttcagcggcgggccgggggagtgctggtgg
cgtcgcacctccagtcttttctcgaagttagctatcgcgtcctcaggcatctcgcccaaccg
tga

Nucleotide sequence of hG-CSF for expression in *W. arrhiza* (the sequence of the signal peptide is highlighted in yellow, stop codon is italicized).

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Expression and Immunogenicity of M2e Peptide of Avian Influenza Virus H5N1 Fused to Ricin Toxin B Chain Produced in Duckweed Plants

Aleksey Firsov[1], Irina Tarasenko[1], Tatiana Mitiouchkina[1], Lyubov Shaloiko[1], Oleg Kozlov[1], Leonid Vinokurov[1], Ekaterina Rasskazova[1], Arkadii Murashev[1], Alexander Vainstein[2] and Sergey Dolgov[1]*

[1] *Institute of Bioorganic Chemistry (RAS), Moscow, Russia,* [2] *Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot, Israel*

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***Correspondence:**
Aleksey Firsov
aleksey_firsov@mail.ru

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The amino acid sequence of the extracellular domain of the virus-encoded M2 matrix protein (peptide M2e) is conserved among all subtypes of influenza A strains, enabling the development of a broad-range vaccine against them. We expressed M2e from avian influenza virus A/chicken/Kurgan/5/2005 (H5N1) in nuclear-transformed duckweed plants for further development of an avian influenza vaccine. The 30-amino acid N-terminal fragment of M2, including M2e (denoted M130), was selected for expression. The M2e DNA sequence fused in-frame to the 3′ end of ricin toxin B chain (RTB) was cloned under control of the CaMV 35S promoter into pBI121. The resulting plasmid was used for duckweed transformation, and 23 independent transgenic duckweed lines were obtained. Asialofetuin-binding ELISA of protein samples from the transgenic plants using polyclonal anti-RTB antibodies confirmed the expression of the RTB–M130 fusion protein in 20 lines. Quantitative ELISA of crude protein extracts from these lines showed RTB–M130 accumulation ranging from 0.25–2.5 μg/g fresh weight (0.0006–0.01% of total soluble protein). Affinity chromatography with immobilized asialofetuin and western blot analysis of protein samples from the transgenic plants showed expression of fusion protein RTB–M130 in the aggregate form with a molecular mass of about 70 kDa. Mice were immunized orally with a preparation of total soluble protein from transgenic plants, receiving four doses of 7 μg duckweed-derived RTB–M130 each, with no additional adjuvant. Specific IgG against M2e was detected in immunized mice, and the endpoint titer of nti-M2e IgG was 1,024. It was confirmed that oral immunization with RTB-M130 induces production of specific antibodies against peptide M2e, one of the most conserved antigens of the influenza virus. These results may provide further information for the development of a duckweed-based expression system to produce a broad-range edible vaccine against avian influenza.

Keywords: duckweed, avian influenza, peptide M2e, ricin B subunit, mice immunization, edible vaccine

INTRODUCTION

Avian influenza is one of the most important diseases in poultry (Swayne et al., 2013). Mass vaccination of domestic and wild birds is the most effective method of preventing this disease (Porter, 2015), hence the importance of inexpensive and easy-to-use vaccines. The ideal vaccine should be able to induce cross-protection against various disease-causing avian influenza strains; it should also be deliverable via mass-immunization methods, such as feeding or in the drinking water; finally, it should be inexpensive to manufacture and convenient for transportation and storage. Plant-produced edible vaccines might be able to meet all of these requirements (Shahid and Daniell, 2016).

Conventional influenza vaccines are based on immunity to the surface viral proteins hemagglutinin and neuraminidase. However, due to antigenic drift, the antigenic properties of these proteins are constantly changing, calling for vaccine updating on a regular basis (Porter, 2015). In this regard, considerable effort is now being focused on the development of vaccines based on conserved viral antigens—the so-called "universal" vaccine—that are effective against many circulating strains. The extracellular domain of matrix protein 2 (M2), termed peptide M2e, and the hemagglutinin stalk region have attracted general interest as antigens for "universal" vaccine design (Oxford, 2013; Wong and Webby, 2013; Hefferon, 2014; Scorza et al., 2016).

Influenza M2 is a transmembrane protein consisting of 97 amino acids; the 23 amino acids at the N terminus form the M2e ectodomain (Pinto and Lamb, 2006). M2 functions as a homotetrameric ion channel and plays an important role in uncoating the virus after its entry. Blocking the ion channel prevents infection of a host cell with the virus (Knipe et al., 2001). In influenza A viruses, the 9 N-terminal amino acids of M2e are completely conserved, while there are only minor changes in the membrane-proximal region. Due to its high conservation among influenza A viruses, M2e has been considered a promising target for inducing cross-protection against different influenza strains (Ito et al., 1991; Fiers et al., 2004; Tumpey et al., 2005). The possibility of developing M2e-based influenza vaccines has been repeatedly confirmed. Some of these vaccines are already in various stages of clinical trials (Oxford, 2013; Zhang et al., 2014; Zheng et al., 2014). Unfortunately, the native form of M2e is poorly immunogenic (Ebrahimi and Tebianian, 2011). One of the strategies for increasing its immunogenicity is to fuse it with adjuvant molecules. The most commonly used adjuvants are hepatitis B core antigen and heat-shock protein 70 of *Mycobacterium tuberculosis* (Oxford, 2013). Other adjuvants, such as *Escherichia coli* flagellin, CD154 from *Salmonella enteritidis* strains and CpG oligonucleotide have also been successfully used to enhance the immunogenicity of the M2e peptide (Ebrahimi and Tebianian, 2011; Oxford, 2013).

Among the promising adjuvants for the development of edible vaccines are plant lectins (Granell et al., 2011; Souza et al., 2013). Plant lectins are a class of carbohydrate-binding proteins; they bind specifically and reversibly to carbohydrate moieties on cell surfaces without biochemical modifications. This ability to bind cell-surface carbohydrate moieties, particularly the M cells on Peyer's patches of mucosal surfaces, has prompted interest in lectins as antigen-delivery agents for mucosal vaccination (Granell et al., 2010). The few experiments involving the immunization of animals with different antigens fused to the nontoxic subunit B of the plant lectin ricin (RTB) have proven the efficacy of RTB as a strong mucosal adjuvant, as low doses of it enhance the immune response to fusion proteins (Choi et al., 2006; Donayre-Torres et al., 2009; Singh et al., 2015). The effectiveness of RTB as an adjuvant is equal to that of subunit B of cholera toxin (Medina-Bolivar et al., 2003). The antigens used in Choi et al. (2006) and Donayre-Torres et al. (2009) were produced in *E. coli*. Medina-Bolivar et al. (2003) and Singh et al. (2015) used antigens expressed in hairy root cultures—tobacco and tomato, respectively. Choi et al.'s (2006) study is the only one in which the antigen was administered orally.

There have been a number of reports on M2e peptide expression in plants (Nemchinov and Natilla, 2007; Denis et al., 2008; Meshcheryakova et al., 2009; Tyulkina et al., 2011; Kang et al., 2012; Petukhova et al., 2014; Mbewana et al., 2015; Tsybalova et al., 2015; Mardanova et al., 2016). In all of them, the M2e was expressed in transient virus vector-based systems. Plant-produced M2e peptide as part of a virus-like particle or fusion protein provided partial (Meshcheryakova et al., 2009; Mardanova et al., 2016) or complete (Denis et al., 2008; Mbewana et al., 2015; Tsybalova et al., 2015) protection of mice against influenza virus challenge. In two of these studies, the M2e peptide was fused to adjuvant proteins—hepatitis B virus core antigen (Tsybalova et al., 2015) or *Salmonella typhimurium* flagellin (Mardanova et al., 2016). In the other experiments, the animals were immunized with virus-like particles that included the M2e peptide but without fusion to the adjuvant protein. The animals in those experiments were immunized parenterally. Since the production of and trials with stably transformed plants are lengthy processes during which vaccines based on hemagglutinin and neuraminidase may lose their relevance, most of these studies were based on transiently expressed antigen. The M2e sequence has remained almost unchanged since 1918 (Tumpey et al., 2005), making it highly suitable for the development of edible vaccines based on nuclear-transformed plants that do not require the expensive industrial-scale facilities necessary for transient expression systems.

Duckweed (*Lemna minor*) is a highly attractive plant system for stable expression of biotechnological products (Gasdaska et al., 2003; Everett et al., 2012). Rapid growth (36 h doubling time) in liquid media, high protein content (up to 45% dry weight), and effective and inexpensive cultivation in bioreactors of various types make this aquatic monocotyledonous plant highly useful for the production of commercially important products in a well-controlled format. The ability to cultivate duckweed in contained systems enables overcoming an important limitation to the commercialization of plant expression systems—potential accidental release of genetically modified plants into the environment during the cultivation of transgenic plants in the field or greenhouse. Several commercially important proteins have already been successfully expressed in duckweed plants: the endoglucanase E1 from *Acidothermus cellulolyticus* (Sun et al., 2007), and monoclonal antibodies

against CD20, CD30, and interferon α2b (Cox et al., 2006). Protective antigen of swine epidemic diarrhea (Ko et al., 2011), tuberculosis antigens ESAT6 and Ag85B (Peterson et al., 2015), and hemagglutinin of avian influenza virus H5N1 (Bertran et al., 2015; Huong et al., 2015) have also been successfully expressed in these plants.

Thus, although the potential of plant-derived RTB as an adjuvant has already been confirmed, the possibility of its oral delivery has not yet been studied. Moreover, the plant-produced M2e was not used for oral immunization in any of the above studies. The aims of this work were to explore the feasibility of expressing M2e fused to RTB (RTB–M130) in nuclear-transformed duckweed plants and to evaluate the immunogenicity of this fusion protein. We report the successful *Agrobacterium*-mediated transformation of duckweed and the expression of peptide M2e as part of the fusion protein RTB–M130 in transgenic plants. We also show that a preparation of partially purified total protein from transgenic plants was immunogenic when ingested, activating an immune response in mice.

MATERIALS AND METHODS

Agrobacterium-Mediated Transformation of Duckweed and Vector Construction

In-vitro culture of duckweed (*L. minor* L.) from Oka River was used in the experiments. *Agrobacterium*-mediated duckweed callus transformation, selection and regeneration of the transformants, and their cultivation *in vitro* were carried out as described previously (Firsov et al., 2015).

A fragment comprising 30 N-terminal amino acid residues of the M2 protein of avian influenza virus A/chicken/Kurgan/5/2005 (H5N1) (GenBank accession no. DQ449633.1), including 23 amino acids of peptide M2e (termed M130), was selected for expression in transgenic duckweed. The DNA sequence of the M130 peptide was optimized for expression in duckweed by codon usage in *Lemna gibba* (http://www.kazusa.or.jp/codon/).

PCR amplification of the RTB-encoding nucleotide sequence (GenBank accession no. X03179) was performed using primers RTB/F and RTB/R (the PCR regimes, primer sequences and cloning sites are presented in Table S1). Total DNA isolated from castor bean leaves was used as the template. The amplified DNA fragment was cloned into the intermediate vector pUC18 and sequenced. The nucleotide sequence of the N-terminal signal peptide of tobacco protein PR1a (GenBank accession no. X12737) was amplified from genomic tobacco DNA using primers RBspF and RBspR. The nucleotide sequence of RTB was cloned in fusion with the 5′ end of M130 and 3′ end of the signal peptide of PR1a into vector pBI121, replacing the β-glucuronidase gene (**Figure 1**). After sequencing, the resulting plasmid (pRTB-M130) was transferred into *Agrobacterium tumefaciens* CBE21 and used for transformation.

In the immunization experiments, we also used transgenic duckweed plants expressing the fusion protein M2e–β-glucuronidase (M2e–GUS; Firsov et al., 2015). Transgenic line G54 with the highest accumulation of M2e–GUS (0.97 mg/g duckweed fresh weight [FW], corresponding to 1.96% of total soluble protein [TSP]) was used. All transgenic lines were grown in 250-ml flasks containing 50 ml of liquid hormone-free MS medium without antibiotics (LHFM medium). Approximately 0.3 g duckweed was inoculated into each flask. The plants were subcultured in fresh medium every 3 weeks. The cultivation of duckweed lines was carried out at 25 ± 1°C, under a 16/8 h photoperiod and illumination intensity of 1.5 W/m^2.

PCR and Southern Blot Analyses

For PCR and Southern blot analyses, the genomic DNA of duckweed was isolated from kanamycin-resistant and non-transformed control plants using the method of Dellaporta et al. (1983). PCR analysis of putatively transgenic plants was performed using primers 5727 and M130r (Table S1). For the Southern blot, duckweed genomic DNA (50 μg) was digested overnight at 37°C with 100 U HindIII which cut the T-DNA of pRTB–M130 at a single position (5′ end of the CaMV 35S promoter). After gel electrophoresis, the digestion products were transferred and immobilized onto Hybond+ membrane (Amersham, USA). The DNA probe was constructed by PCR using plasmid pRTB–M130 as the template and primers RBspF and M130r, amplifying the nucleotide sequence of fusion protein RTB–M130. Probe DNA (1,003 bp) was labeled with alkaline phosphatase using the AlkPhos Direct Labeling Kit (Amersham Bioscience, USA). Prehybridization, hybridization (overnight at 60°C) with alkaline phosphatase-labeled probe, and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling Kit protocol. Detection was performed using CDP-Star detection reagent following the manufacturer's directions (Amersham Bioscience).

Asialofetuin-Binding ELISA

RTB–M130 recombinant protein was quantified in protein extracts of transgenic duckweed plants by asialofetuin-binding assay (Dawson et al., 1999). TSP was extracted immediately prior to the analysis. Duckweed plants (1.0 g) were ground in liquid nitrogen. The ground material was resuspended in an equal volume of phosphate buffer saline (PBS, pH 7.4) containing 0.05% (v/v) Tween 20 (PBST buffer). Total proteins were extracted for 20 min at 4°C, then centrifuged for 10 min at 16,000 g, 4°C and the supernatant was taken for further analysis. Protein concentration was measured by DC protein assay (BioRad, USA).

Analyzed protein samples (100 μl/well) were added to the wells with immobilized asialofetuin. Rabbit anti-RTB polyclonal antibody (AbCam, UK) was used as the primary antibody, and anti-rabbit IgG conjugated to alkaline phosphatase (Pierce, USA) as the secondary antibody. Antibodies were diluted in blocking buffer, 1:1,000 for primary and 1:2,000 for secondary antibodies. Phosphatase Substrate Kit (Pierce) was used, and the reaction was performed as per the manufacturer's instructions. The reaction was stopped after 20 min at 25°C and absorbance was measured at 405 nm.

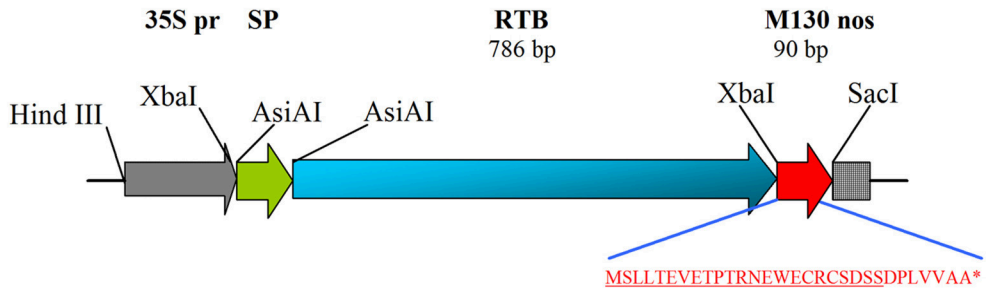

FIGURE 1 | Schematic depiction of the expression cassette of plasmid pRTB–M130. The amino acid sequence of M130 peptide is shown. 35S pr, CaMV 35S promoter; SP, N-terminal signal peptide of tobacco PR1a protein; RTB, subunit B of ricin; M130, N-terminal fragment of the M2 protein of avian influenza virus containing peptide M2e (underlined); nos, nopaline synthase terminator. The sizes of RTB and M130 are indicated. *Denotes a stop codon, i.e., end of the amino acid sequence of RTB-M130 protein.

Ricin, extracted from castor seeds following Waller and Negi's (1958), was used as a standard for the quantification of RTB–M130 accumulation in transgenic plants. The amount of plant-expressed RTB–M130 was estimated based on reference ricin standards taking into account differences in their molecular mass (32.6 kDa for RTB–M130 and 64.2 kDa for ricin).

Affinity Chromatography with Immobilized Asialofetuin

Affinity chromatography with immobilized asialofetuin was carried out by the method described in Donayre-Torres et al. (2009). In this experiment, we used transgenic line 81 and non-transformed duckweed as a negative control. The duckweed plants (30 g FW) were ground in liquid nitrogen. The ground material was resuspended in three volumes of extraction buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol and protease inhibitor cocktail (Sigma). Total proteins were extracted for 40 min at 4°C, then centrifuged for 30 min at 11,000 *g* at 4°C and the supernatant was taken for analysis.

The proteins were precipitated from the crude extract by ammonium sulfate (pH 7.8). Ammonium sulfate was added to samples to a concentration equal to 30% saturation followed by incubation for 12 h at 4°C and centrifugation at 11,000 *g* for 30 min. The precipitate was discarded and ammonium sulfate was added to the supernatant to 70% saturation followed by incubation and centrifugation as indicated above. The obtained precipitate was collected, dissolved in PBS and desalted on a Sephadex G25 column. The obtained preparation was loaded onto an asialofetuin–Sepharose 4B column equilibrated with PBS (pH 7.4). The proteins that bound to the asialofetuin–Sepharose were eluted in 200 mM glycine (pH 3.65) and five fractions were collected. These fractions were analyzed by western blotting.

Western Blot Analysis

The proteins were separated by 10–25% gradient SDS-PAGE and transferred to a nitrocellulose membrane (Amersham). Rabbit anti-RTB polyclonal antibodies (diluted 1:1,000) served as the primary antibodies. Anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody (1:4,000). Blots were treated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for visualization.

Enrichment of Crude Soluble Protein Extracts from Transgenic Plants for Immunization Assay

The extraction and ammonium sulfate precipitation of TSP from transgenic duckweed lines 81 and G54 were performed as described above for chromatography with immobilized asialofetuin. Precipitated proteins from line 81 were dissolved in PBS buffer and desalted on a Sephadex G25 column equilibrated with 20 mM ammonium bicarbonate buffer (pH 8.2). RTB–M130 protein was quantified in the obtained preparation using asialofetuin-binding ELISA.

The pellets from duckweed line G54 were dissolved in 20 mM Tris–HCl (pH 8.0) and 1 M NaCl, and loaded without desalting onto a Toyopearl butyl chromatography column equilibrated with the same buffer. The proteins were eluted by an inverse linear gradient of NaCl (1.0–0 M) in 20 mM Tris–HCl (pH 8.0). The column was then washed with water, eluting the fusion protein M2e–GUS. M2e–GUS protein was quantified in the obtained preparation by ELISA. The preparations of RTB–M130 and M2e–GUS were then aliquoted and lyophilized and the samples were stored at −70°C until immunization assay.

Immunization Assay

Adult (7- to 9-week-old) male ICR mice weighing 32 ± 6 g were used for the immunization assay. This animal study was conducted in compliance with Good Laboratory Practice (GLP certificate no. G-044), EC Directive 2010/63/EU, and the Russian legislation regulating animal experiments in laboratories for biological testing. Mice were divided into three groups: (1) immunization with RTB–M130 (7 μg/dose, 10 animals); (2) immunization with M2e–GUS (28 μg/dose, 10 animals); and (3) administration of TSP from non-transformed plants (100 μg/dose, 5 animals). Immediately before the assay, immunization preparations were resuspended in sterile PBS and homogenized for 5 min in an ultrasonic bath. The doses (volume 200 μl) were orally administered using a 1-ml syringe equipped with a bulb-tipped gastric gavage needle. The immunizations were carried out

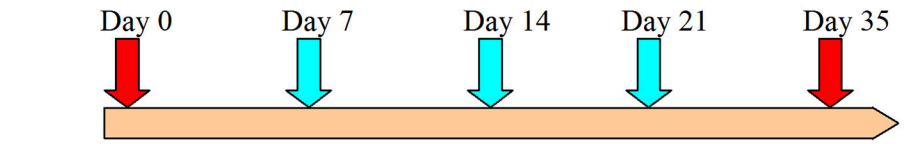

FIGURE 2 | Immunization assay design. The turquoise arrows indicate oral gavage immunizations, red arrows, day 0 and blood samples; K-, denotes non-transformed duckweed plants.

on days 0, 7, 14, and 21 (**Figure 2**). Blood samples were collected 14 days after the fourth immunization, i.e., on day 35, from the mouse's retro-orbital sinus. To evaluate the basal mouse serum response, blood was also collected on day 0, before initiating the oral immunizations.

ELISA was performed to evaluate the immunization response. The microtiter plates were coated with conjugate peptide M2e–keyhole limpet hemocyanin (2 μg/well in PBS) at 4°C for 2 h, then washed and blocked in PBST with 2% (w/v) bovine serum albumin. The mouse sera were initially diluted 32 times in PBS with a subsequent two-fold serial dilution. The plates were incubated with 100 μl/well of mouse serum for 16 h at 4°C. The plates were then washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG (dilution 1:5,000, BioRad) for 2 h at 4°C. The Horseradish Peroxidase Substrate Kit (BioRad) was used, and the reaction was performed as per the manufacturer's instructions. The reaction was stopped after 45 min at room temperature and absorbance was measured at 450 nm. Endpoint antibody titers were defined as the last serum dilution at which the absorption at 450 nm was significantly higher than that of serum of mice immunized with non-transformed plant TSP. Results are expressed as mean optical density (OD) ± *SD* for groups of mice.

Statistical Analysis

The significance of differences in RTB–M130 accumulation between duckweed transgenic lines and in mean antibody levels between groups of immunized mice were analyzed by one-way analysis of variance (ANOVA) test (Statistica 6.1 software; StatSoft Inc).

RESULTS

Agrobacterium-Mediated Transformation of Duckweed

Duckweed plants were transformed with *A. tumefaciens* strain CBE21 carrying the RTB–M130 construct encoding M2e peptide translationally fused to the 3′ end of RTB (**Figure 1**); 10 g of duckweed callus was used for *Agrobacterium*-mediated transformation. First regenerated fronds, following transformation and regeneration, appeared after 10–12 weeks of cultivation on the medium for transformant selection. Each frond was transferred to a separate culture tube with LHFM medium containing 10 mg/l kanamycin and 200 mg/l cefotaxime for further growth and proliferation. During the cultivation, all plants showing signs of the toxic effect of kanamycin were culled. As a result, 23 independent kanamycin-resistant duckweed lines were obtained. The kanamycin-resistant duckweed plants did not differ morphologically from the non-transformed ones (**Figures 3A,B**). The development and growth rate of these plants in liquid culture did not differ from the corresponding characteristics of the non-transformed control plants.

Analysis of RTB–M130 Integration and Expression in Transgenic Plants

The kanamycin-resistant duckweed lines were analyzed by PCR for the presence of the target RTB–M130 sequence. A DNA fragment of the expected size was amplified from the DNA of all 23 analyzed lines. In DNA samples from non-transformed plants, amplification of the target fragment was not observed. To further confirm the transgenic origin, Southern blot analysis of selected duckweed lines was performed. Genomic DNA was digested with HindIII that cut once within the T-DNA prior to electrophoresis. Results confirmed integration of the nucleotide sequence encoding the RTB–M130 fusion protein into the duckweed genomic DNA (**Figure 4**). Based on the hybridization profile, there were two (line 91) or three (lines 81, 83, and 101) insertions of the transgene in the studied lines, which is typical for *Agrobacterium*-mediated transformation. The DNA from non-transformed plants failed to hybridize to the probe.

Asialofetuin-binding ELISA revealed the presence of ricin B subunit in all analyzed transgenic duckweed lines (**Figure 5**). In three transgenic lines (19, 24, and 43), only negligible amounts of RTB were detected. The accumulation of the fusion protein RTB–M130 in transgenic lines varied from 0.25 μg/g (line 26) to 2.5 μg/g FW (line 81), corresponding to 0.0006 and 0.01% of TSP,

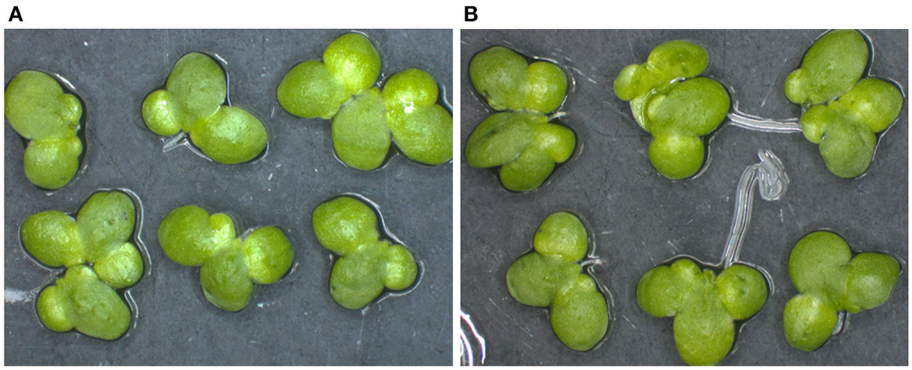

FIGURE 3 | Transgenic and non-transformed duckweed plants. **(A)** Transgenic duckweed plants (line 81) growing in LHFM medium with 10 mg/l kanamycin. **(B)** Non-transformed duckweed plant growing in kanamycin-free medium.

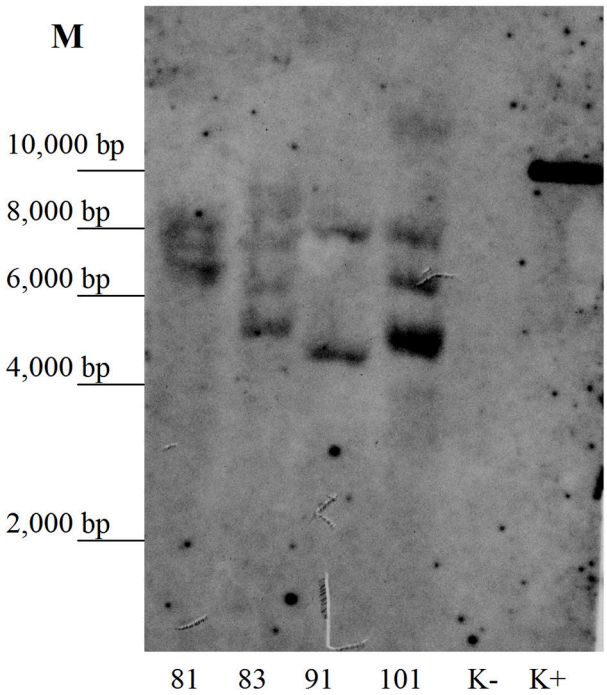

FIGURE 4 | Southern blot analysis of the primary transformants**.** Numbers denote independent transgenic lines. K–, DNA from non-transformed plants; K+, DNA of plasmid pRTB–M130; M, molecular size markers.

respectively. A relatively high level of RTB–M130 expression was observed in lines 81, 83, 91, and 101—about 2.0 μg/g FW (0.01% of TSP). Based on the obtained data, we selected transgenic lines with high and average levels of accumulated product for further studies (lines 41, 60, 81, 83, 91, and 101).

Western blot analysis of total protein samples from the selected transgenic duckweed lines revealed the presence of an immunoreactive band of ∼70 kDa (**Figure 6A**). The 32-kDa bands corresponding to the fusion protein RTB–M130 were not observed. Immunoreactive bands of these weights were not detected in the protein samples from non-transformed control plants. An additional experiment was performed to evaluate the interaction between RTB–M130 and asialofetuin by affinity chromatography (**Figure 6B**). Western blot analysis of protein fractions from transgenic line 81, obtained following affinity chromatography with immobilized asialofetuin, using antibodies to RTB, revealed the presence of an immunoreactive band at around 70 kDa. The corresponding bands were not detected in protein fractions from non-transformed plants. From these results, we hypothesized that RTB–M130 is expressed as an aggregate with a molecular mass of about 70 kDa. Recombinant RTB–M130's ability to bind to the glycoprotein asialofetuin suggested its ability to bind to cellular receptors.

Mouse-Immunization Assay

In a preliminary experiment, laboratory mice were reluctant to eat duckweed—fresh or lyophilized, alone or mixed with dry feed. This made it impossible to immunize the mice by feeding duckweed biomass. To overcome this, immunization with partially purified preparations of TSP from transgenic duckweed was performed by gastric gavage.

As expected, in mice immunized with TSP from non-transgenic plants, no antibody to M2e was detected. Moreover, no animals had detectable levels of M2e-specific IgG in their preimmune serum, indicating that there had been no prior exposure to the influenza virus. Plant-derived RTB–M130 and M2e–GUS fusion proteins elicited a specific immune response (**Figure 7**). IgG against peptide M2e accumulated in all mice immunized with RTB–M130 and M2e–GUS, indicating that the fusion proteins are internalized through the mucosal lining and able to induce the immune system.

As shown in **Figure 7**, mice gavaged with RTB–M130 developed specific anti-M2e IgGs with an endpoint ELISA titer of 1024. Immunization of animals with M2e–GUS also elicited an immune response, with an anti-M2e IgG titer of 512. The immune response in this group was markedly weaker, even though each mouse received 112 μg M2e–GUS in its immunization cycle, compared to 28 μg/mouse of RTB–M130. The obtained results confirmed retention of the plant-derived

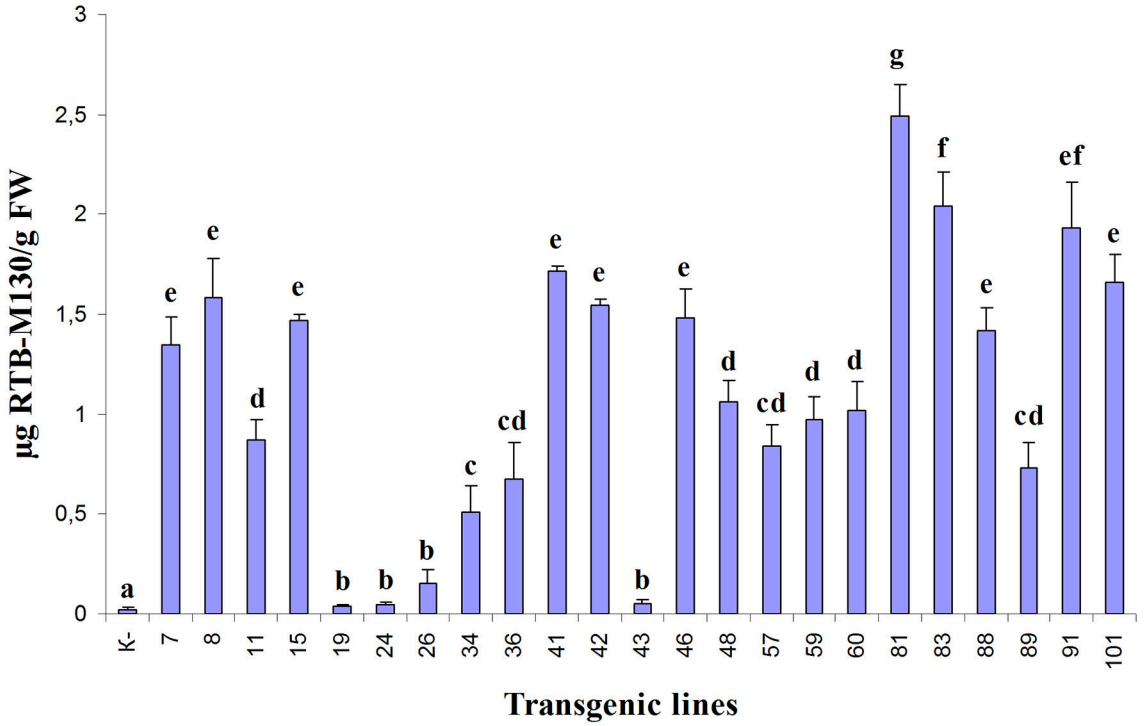

FIGURE 5 | Quantification of RTB–M130 fusion protein in transgenic duckweed plants by asialofetuin-binding ELISA using anti-RTB antibody. K–, non-transformed plants. Numbers denote transgenic lines. Error bars indicate ± *SD*. Different letters denote significant differences for mean amount of RTB–M130 among duckweed transgenic lines ($P < 0.05$). Quantification of RTB–M130 was performed in three biological replicates.

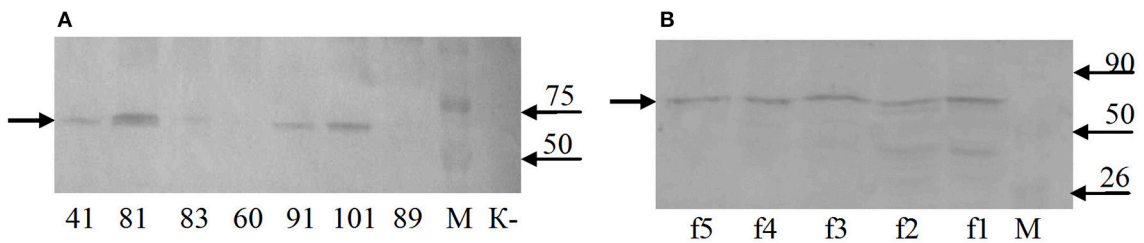

FIGURE 6 | RTB–M130 protein expression in transgenic duckweed lines. **(A)** Western blot analysis of protein samples from plants. **(B)** Western blot analysis of fractions after chromatography with immobilized asialofetuin (transgenic line 81). K–, non-transformed duckweed plants; M, molecular size markers. Numbers denote transgenic lines; f1–f5, chromatography fractions (transgenic line 81). Arrow on the left indicates immunoreactive band with molecular mass of ~70 kDa.

RTB's adjuvant properties, and weak immunogenicity of peptide M2e with GUS as the adjuvant. In summary, these results confirm the possibility of expressing M2e peptide in nuclear-transformed duckweed and reveal a much higher level of anti-M2e IgG in orally immunized mice when M2e is fused to RTB as compared to GUS.

DISCUSSION

We obtained 23 different lines of duckweed with confirmed transgenic status; the fusion protein RTB–M130 was detected in all of these lines. Transgenic duckweed plants did not differ morphologically from their non-transformed counterparts. Expression of the fusion protein had no effect on growth rate in liquid culture or on plant TSP content. Accumulation of RTB–M130 according to asialofetuin-binding ELISA ranged from 0.25 to 2.5 µg/g FW of duckweed (0.0006–0.01% of TSP). Strong variations in recombinant protein expression in independently derived transgenic lines are common (Richter et al., 2000; Sharma and Sharma, 2009). They are often related to differences in the number of transgene copies or in the genome position into which the foreign DNA has integrated. Both of these factors are relevant to the transgenic duckweed lines that we obtained.

A few studies have been performed on the expression of RTB in heterologous systems, alone or as part of a fusion protein. According to Reed et al. (2005), the accumulation of ricin B

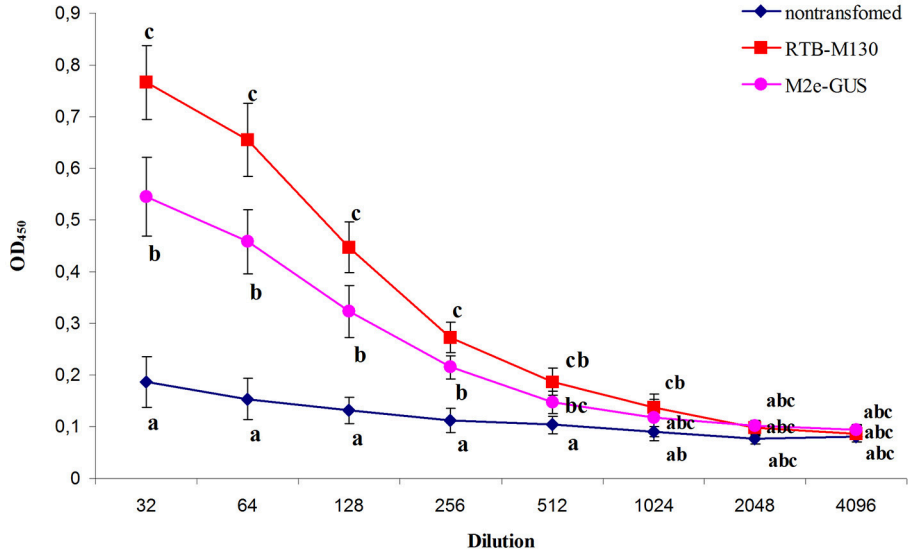

FIGURE 7 | Immune response of mice after oral immunization with total soluble protein from RTB–M130- and M2e–GUS-transgenic duckweed. Total protein from non-transgenic plants was used as a control in the immunization experiments. Levels of IgG against M2e were evaluated by ELISA. Results are presented as mean OD values ± *SD*. Different letters indicate significant differences between immunized groups ($P < 0.05$).

subunit in transgenic tobacco plants averaged 0.007% of TSP (about 0.35 μg RTB functional equivalent per 1 g leaf FW). In Woffenden's, Ñopo, Cramer, Dolan and Medina-Bolivar (2008), tobacco plants were transformed with the nucleotide sequence encoding RTB in translational fusion with synthetic genes F1 and V of *Yersinia pestis*. The fusion protein RTB–F1–V accumulated in the leaves of transgenic plants at a level of about 45 ng RTB functional equivalent per 1 g FW (corresponding to 0.0015–0.0025% of TSP). Somewhat higher levels of RTB expression were observed by Choi et al. (2006)—up to 0.03% of TSP. In that study, ricin B subunit was fused to the C-terminal end of capsid glycoprotein VP7 of monkey rotavirus SA11, and fusion protein VP7–RTB was expressed in potato tubers. Transformation of tobacco with cDNA encoding the full-length preproricin resulted in its accumulation in leaf tissues to 0.25% of TSP (Sehnke et al., 1994). In those studies, asialofetuin-binding ELISA showed that the ricin B subunit was functionally active, indicating its correct processing and glycosylation in the heterologous systems. Interestingly, in Singh et al. (2015), the accumulation of fusion protein rabies glycoprotein (RGP)–RTB in tomato hairy roots reached 1.14% of TSP. RGP–RTB was expressed as a monomer; its functional viability was confirmed by asialofetuin-binding ELISA.

It should be noted that the accumulation of GUS in line G54 was almost a thousand times greater than that of RTB–M130 in our transgenic lines. A high level of GUS accumulation is fairly common. For example, in a study by Butaye et al. (2004), GUS accumulation in the cytoplasm reached 10% of TSP. It seems that there, the high expression of GUS was due to its high stability in the cytoplasm (half-life in living mesophyll protoplasts of ∼50 h; Jefferson et al., 1987), allowing it to accumulate in large quantities.

A low level of accumulation seems to be characteristic of RTB expressed in nuclear-transformed heterologous systems. This is most likely due to its rapid degradation in the secretory pathway of plant cells. In experiments with tobacco (both protoplasts and nuclear-transformed plants), the bulk of the newly synthesized RTB disappeared in the early secretory pathway, presumably as a result of proteolysis in the endoplasmic reticulum. The recombinant RTB degraded very rapidly, and its accumulation in the protoplasts was therefore very low (Chamberlain et al., 2008). The plant cell proteolytic system likely recognizes the recombinant RTB as a misfolded or unassembled polypeptide that is then targeted for elimination. Kiani et al. (2013) observed a high level of RTB expression in transgenic cotton plants. In those experiments, RTB was fused to the C terminus of the Cry1Ac protein (a δ-endotoxin of *Bacillus thuringiensis*). The fusion protein Cry1Ac–RTB was targeted to chloroplasts by a transit peptide from petunia, and its accumulation in the plants was estimated at 3.5% of TSP. The high level of accumulation of Cry1Ac–RTB might have been because it was transported into chloroplasts without entering the endoplasmic reticulum, thereby avoiding degradation.

In our experiments, fusion protein RTB–M130 was detected as a band with a molecular mass of about 70 kDa (calculated mass of the protein without the N-terminal signal peptide–32.6 kDa), and it was therefore presumably in aggregated form. Similar expression behavior of RTB in a fusion protein was observed by Carter et al. (2010), where it was fused with the C-terminal end of proinsulin (Ins) in transgenic potato tubers. Those authors showed that the fusion protein Ins–RTB apparently aggregates in large molecular complexes, being detected as bands of more than 200 kDa (estimated mass of Ins–RTB-−38.2 kDa). Attempts to dissociate these aggregates into monomers were unsuccessful.

The authors believed that aggregation of Ins–RTB occurs due to the formation of a large number of chaotic intermolecular disulfide bonds. Nevertheless, Ins–RTB retained the ability to bind to asialofetuin (Carter et al., 2010). In our experiments, RTB–M130 also retained the ability to bind to asialofetuin and elicit an immune response in mice. Thus, aggregation of RTB does not influence its ability to bind to its corresponding cell receptors and act as an adjuvant.

Aggregation has only been observed when RTB is fused with small proteins—M130 peptide (3.3 kDa) or proinsulin (9.4 kDa). In experiments where RTB is fused with a relatively large protein, e.g., VP7 (34 kDa; Choi et al., 2006), F1 and V proteins (15 and 37 kDa, respectively; Woffenden et al., 2008), green fluorescent protein (26 kDa; Medina-Bolivar et al., 2003), RGP (56.5 kDa; Singh et al., 2015), Cry1Ac protein (65 kDa; Kiani et al., 2013), or expressed as ricin alone (Sehnke et al., 1994), there is no aggregation, and fusion proteins have the expected molecular mass. Size and structure optimization of the RTB partner in fusion proteins may prevent or substantially reduce its aggregation when expressed in heterologous systems.

As already noted, the M2e peptide is a poor immunogen, and requires an adjuvant to become sufficiently immunogenic (Rossman and Lamb, 2011). In most cases, the M2e peptide is expressed in heterologous systems in fusion with some adjuvant protein. To obtain a mucosal immune response, such adjuvants have included CTA1–DD (subunit A of cholera toxin fused to the D domain of protein A from *Staphylococcus aureus* (De Filette et al., 2005, 2006), subunit B of cholera toxin (Shim et al., 2011), hepatitis B virus core antigen (Ravin et al., 2012), and C-terminal domain of heat-shock protein 70 from *M. tuberculosis* (Ebrahimi and Tebianian, 2011).

The immunogenicity of the RTB–M130 fusion protein and the adjuvant effect of RTB were confirmed in our study. Immunization of mice with RTB–M130 elicited a detectable immune response. Immunization with M2e–GUS elicited a weaker immune response, even though the antigen dose was significantly higher than when immunized with RTB–M130. When the mice were immunized with RTB–M130, the anti-M2e antibody titer was clearly insufficient for complete protection against the influenza virus. We suggest increasing the immunization dose of RTB–M130. To this end, it is necessary to increase the level of RTB–M130 accumulation in transgenic plants. We believe that this can be achieved by (1) optimizing the size and structure of the antigenic part in the fusion protein and (2) selecting the optimal compartment for its accumulation.

Our experiments demonstrated the feasibility of expressing M2e peptide fused to ricin B subunit in nuclear-transformed duckweed plants, with no impact on plant morphology or growth rate. Oral immunization with the recombinant RTB–M130 protein elicited an immune response and induced anti-M2e antibodies in mice. The immunization assay also confirmed that the ricin B subunit retains its adjuvant properties in the fusion protein, enhancing induction of anti-M2e antibodies in the vaccinated animals. The present study demonstrates that transgenic duckweed plants can produce quality antigen toward the development of an edible "universal" vaccine against influenza viruses.

AUTHOR CONTRIBUTIONS

TM and IT: Performed genetic transformation and *in vitro* duckweed culture; LS, OK, and LV: Analyzed target protein expression, including western blotting and chromatography, and prepared the samples for mouse immunizations; AF and IT: Performed the PCR and Southern blot analyses; AM and ER: Performed the immunization assays; SD and AV: Planned the experiments, provided general guidance, and prepared the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00022/full#supplementary-material

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