REDISCOVERING LOCAL LANDRACES: SHAPING HORTICULTURE FOR THE FUTURE

EDITED BY: Spyridon A. Petropoulos, Isabel C. F. R. Ferreira and Lillian Barros
PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-847-9
DOI 10.3389/978-2-88945-847-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

REDISCOVERING LOCAL LANDRACES: SHAPING HORTICULTURE FOR THE FUTURE

Topic Editors:
Spyridon A. Petropoulos, University of Thessaly, Greece
Isabel C. F. R. Ferreira, Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Portugal
Lillian Barros, Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Portugal

Images: Dr. Petropoulos Spyridon.

Local landraces are traditional crop varieties cultivated in specific locations. However, the intensification of modern horticulture has put these genotypes aside, since farmers tend to select hybrids or commercial cultivars due to higher yield, uniformity and marketability.

The various landraces are very distinct in their quality features, therefore it is of high importance to highlight these differences and identify genotypes that could be further exploited by producing high added value products and by reinforcing local rural economies.

The proposed Research Topic aims to reveal the importance of local landraces for sustainable horticulture, focusing on their special quality features as the result of adaptation to specific growing conditions after domestication.

Citation: Petropoulos, S. A., Ferreira, I. C. F. R., Barros, L., eds. (2019). Rediscovering Local Landraces: Shaping Horticulture for the Future. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-847-9

Table of Contents

frontiers in Plant Science

EDITORIAL
published: 12 February 2019
doi: 10.3389/fpls.2019.00126

Editorial: Rediscovering Local Landraces: Shaping Horticulture for the Future

Spyridon A. Petropoulos[1]*, *Lillian Barros*[2] *and Isabel C. F. R. Ferreira*[2]*

[1] *Laboratory of Vegetable Production, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Volos, Greece,* [2] *Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Bragança, Portugal*

Keywords: biodiversity, common bean, fruit quality, horticulture, genetic resources, legumes, lettuce landraces, tomato landraces

Editorial on the Research Topic

Rediscovering Local Landraces: Shaping Horticulture for the Future

OPEN ACCESS

Edited by:
Angeles Calatayud, Instituto Valenciano de Investigaciones Agrarias, Spain

Reviewed by:
Maria Jose Diez, Universitat Politècnica de València, Spain
Cristina Mallor, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Spain

***Correspondence:**
Spyridon A. Petropoulos
spetropoulos@uth.gr
Isabel C. F. R. Ferreira
iferreira@ipb.pt

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *24 November 2018*
Accepted: *24 January 2019*
Published: *12 February 2019*

Citation:
Petropoulos SA, Barros L and Ferreira ICFR (2019) Editorial: Rediscovering Local Landraces: Shaping Horticulture for the Future. Front. Plant Sci. 10:126. doi: 10.3389/fpls.2019.00126

Local landraces constitute a valuable genetic pool of increased diversity that can be exploited in breeding programs for the production of new commercial cultivars with targeted traits. Despite the acknowledged importance of local landraces, recent market trends and modern horticultural production systems have shifted farmers and related sectors to cultivation of commercial cultivars and hybrids due to their high yield and uniformity of the final product. However, this trend results only to short-term benefits while it puts in danger the valuable genetic resources of local horticultural landraces which may be proven useful under a global climate change. Moreover, consumers' concerns about food quality, its origin and production process as well as geographical indication (GI) of products are in increasing demand. Especially for the EU, Protected Designation of Origin (POG), Protected Geographical Indication (PGI) and Traditional Specialties Guaranteed (TSG) labels have been established, while according to the TRIPS (Trade Related Aspects of Intellectual Property Rights) agreement which is applicable to the WTO (World Trade Organization) participating countries other labels such as Certification Mark and Collective Mark are also available for non-EU countries. Within this context and considering the special features that characterize local landraces and the cultural heritage and connection with specific regions, it is of pivotal importance to further investigate and point out these features that could result in high added value products and the reinforcement of rural economies.

Therefore, the prospect of valorization of landraces and traditional crop varieties seems very promising toward a sustainable and quality-orientated horticulture.

This e-book aims to highlight the significance of conserving local landraces toward shaping future horticultural production in a sustainable framework and point out all these special features that characterize local landraces and make them valuable, especially under harsh conditions and/or abiotic and biotic stress factors. In this context, Major et al. tried to differentiate 13 shallot landraces preserved along the Croatian coast by using morphological characters, while the authors also studied the chemical composition of the tested landraces. After using a multivariate classification, it was suggested that Croatian shallot landraces can be classified into three distinct groups (*Allium cepa* Aggregatum, *A.* x *proliferum*, and *A.* x *cornutum*), while biochemical profiles and morphological parameters could be used for species identification. "Calçots" are a typical Spanish product which is produced by immature floral stems of onion landrace "Blanca Tardana de Lleida" resprouts. In the study of Sans et al., the effect of pre-harvest factors (genotype and growing conditions) on chemical composition and sensorial properties of calçots was evaluated by assessing the original genotype and three new genotypes derived from the original one under

different conditions. Based on the results, modulation of cultivation practices, growing conditions, and breeding selection may increase yield and quality of the final product. Another landrace that is described in this e-book is "Carota di Polignano," a multi-colored Italian landrace of carrot which was evaluated for the possibility of biofortifying with iodine by Signore et al. under open field and greenhouse conditions. The results of that study demonstrated that open field cultivation allows for better iodine enrichment of the final product, thus Recommended Daily Allowance may be achieved easier through the consumption of lower amounts of these carrots.

Tomato is the most important fruit vegetable and many local landraces are preserved throughout the world as local or farmer varieties. In their study, Massaretto et al. evaluated the potential of using two tomato landraces (Negro Yeste and Verdal), endemic of the Spanish Southeast area which is characterized by harsh climatic conditions, as alternatives to commercial varieties for cultivation under saline growing conditions. High adaptability of local landraces to salinity was observed, since both landraces exhibited high fruit quality and yield comparing to the commercial cultivar tested under the same conditions. Similarly, Figás et al. evaluated 12 long shelf-life tomato genotypes, including seven landraces, for suitability under open field and protected environment growing conditions. The variation in chemical composition, plant morphology, and agronomic performance revealed that growing conditions have a high impact on shelf-life of tomato fruit. In that way, landraces could be a useful material in breeding programs focusing on adaptation of tomato genotypes to greenhouse cultivation.

On the other hand, the interest in leafy vegetable landraces is also discussed in the present e-book. Casals Missio et al. evaluated agronomic and quality features as well genetic variability of 32 lettuce genotypes, including landraces and modern varieties, in organic farming agrosystems. Moreover, farmers and consumers participated in genotype characterization in terms of yield, susceptibility to *Bremia lactucae* infections, visual appearance, and taste. According to the results, modern lettuce varieties and landraces were clearly distinguished, mostly due to significant differences in marketable weight and tolerance to infestations by *B. lactucae*, while farmers and consumers showed high capacity for phenotype characterization and they should be included in future research projects for local landraces recovery. Moreover, Renna et al. performed studies of nutritional value, mineral composition and antioxidant activity evaluation, as well as ethnobotanical surveys of various unconventional vegetables traditionally used in Puglia (Southern Italy). The studied vegetables included offshoots of two globe artichoke landraces, greens of summer squash and faba beans landraces, and crenate broomrape. Two more research articles and two mini reviews highlighted the importance of genetic diversity of legume germplasm. In particular, Cullis et al. presented the features that allow marama bean, an orphan legume indigenous to Southern Africa (Kalahari Desert), to survive under harsh conditions. It was also suggested the importance of unveiling the acclimatization mechanisms to improve the performance of major crops under a climate change regime. The next mini review authored by De Ron et al. addressed the importance of conservation of common bean, runner bean, and cowpea germplasms for the genetic improvement of varieties toward the sustainable production of legumes. The recovery of "Caparrona" or "Caparrona de Monzón" common bean landrace for commercial purposes concerned Mallor et al. who performed both *in situ* and *ex situ* experiments after collection of seed samples of the aforementioned landrace. From all the collected seed samples, only two of them were finally used to register and commercialize "Caparrona" beans as a gourmet product by a local producers' association. In the next original research article authored by Rivera et al., a Spanish core collection of 202 common bean landraces was evaluated in terms of chemical composition, showing a high genetic diversity. The results also showed no significant correlation between chemical composition and sensorial quality which could be further valorized in the development of elite cultivars with superior nutritional composition and sensory traits. Finally, Marconi et al. performed a genetic characterization of 175 apple accessions from Central Italy, including local, modern, and ancient varieties, by using 19 Single Sequence Repeats (SSR) markers. The results showed a significant genetic variation among the tested accessions, while duplicates, synonyms, and homonyms were also identified.

Modern horticulture faces serious challenges due to climate change and the increasing market demands for food quality and food safety. Local landraces could be proven a useful tool toward shaping a sustainable and quality-oriented horticulture. We believe that the present e-book will raise the scientific interest to local landraces of horticultural species in order to reveal their importance to crop adaptation to the ongoing climate change, as well as their socio-economic impact on rural communities. We also believe that future research concerning local landraces of horticultural species is of pivotal importance in order to unveil their special features, to evaluate these genotypes under intensified cultivation systems and to include them in breeding programs for the production of new elite genotypes.

AUTHOR CONTRIBUTIONS

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

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 December 2018
doi: 10.3389/fpls.2018.01749

Morphological and Biochemical Diversity of Shallot Landraces Preserved Along the Croatian Coast

Nikola Major[1], Smiljana Goreta Ban[1,2], Branimir Urlić[3], Dean Ban[1,2], Gvozden Dumičić[4] and Josipa Perković[1]*

[1] Department of Agriculture and Nutrition, Institute of Agriculture and Tourism, Poreč, Croatia, [2] The Centre of Excellence for Biodiversity and Molecular Plant Breeding, Zagreb, Croatia, [3] Department of Applied Sciences, Institute for Adriatic Crops and Karst Reclamation, Split, Croatia, [4] Department of Plant Sciences, Institute for Adriatic Crops and Karst Reclamation, Split, Croatia

Shallots are a valuable minor *Allium* crop, and are propagated vegetatively and maintained in home gardens across generations along the Croatian coast and island areas. Shallot landraces growing along the Croatian coast fall into three genotypes: *Allium cepa* Aggregatum group ($2n = 2x = 16$), *A.* × *proliferum* (Moench) Schard. ($2n = 2x = 16$), and *A.* × *cornutum* Clementi ex Vis. ($2n = 3x = 24$), among which *A.* × *cornutum* is the most widespread. The aim of this study was to differentiate shallot accessions collected from local farmers using morphological markers. Also, the chemical composition including phenolic content, phenolic profile, total antioxidant capacity, and mineral composition, of shallot accessions was compared with that of the local landraces of common onion, and with market available shallot and common onion cultivars. Based on morphological observations and using multivariate classification, shallot landraces were classified into three distinct groups. Properties, based on which *A.* × *cornutum* can be differentiated from *A. cepa* Aggregatum and *A.* × *proliferum,* are stamen morphology, stamen length, leaf and scape vegetative properties, number of bulbs in cluster, cluster mass, and bulb diameter. Flower diameter and flower pedicel length differentiate *A.* × *cornutum* and *A.* × *proliferum* from *A. cepa* Aggregatum. Significant variability was observed in the biochemical profiles across tested accessions. Compared with the commercial common onion cultivars, local shallot accessions have higher bulb N, P, and K content. The major phenolic compounds identified in shallots were quercetin-4′-glucoside and quercetin-3,4′-diglucoside. Additionally, several other minor phenolic compounds were also identified. Morphological and biochemical profiles were evaluated using Partial Least Square (PLS) analysis. Specific morphological traits and biochemical markers for possible species identification are proposed.

Keywords: landrace, mineral composition, morphology, shallot, phenols, PLS

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Maria Gonnella, Consiglio Nazionale delle Ricerche, Italy
Antonio Tiberini, Università Mediterranea di Reggio Calabria, Italy

****Correspondence:***
Smiljana Goreta Ban
smilja@iptpo.hr

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *13 July 2018*
Accepted: *09 November 2018*
Published: *03 December 2018*

Citation:
Major N, Goreta Ban S, Urlić B, Ban D, Dumičić G and Perković J (2018) Morphological and Biochemical Diversity of Shallot Landraces Preserved Along the Croatian Coast. Front. Plant Sci. 9:1749. doi: 10.3389/fpls.2018.01749

INTRODUCTION

Allium is a taxonomically complicated genus with more than 750 species, and approximately 60 taxonomic groups at subgenera, sectional, and subsectional ranks (Ohri et al., 1998; Fritsch and Friesen, 2002; Block, 2010). Based on inflorescence morphology, *Allium* was once classified as *Liliaceae* and later as *Amaryllidaceae* (Block, 2010). Recently, molecular data have supported further subdivision into small monophyletic families (Fritsch and Friesen, 2002), and placement of *Allium* and its close relatives in the *Alliaceae* family (Takhtajan, 2009).

The origin of the *Allium* spp. is still somewhat a mystery and many botanists doubt the existence of *Allium cepa* as a wild plant (Pike, 1986). Domestication of *Allium* occurred more than 4000 years ago, with spread to Egypt, ancient China, and Persia (Fritsch and Friesen, 2002; Ansari, 2007; Cumo, 2015). *Allium* is currently widely distributed in Europe, Central Asia, North America, and India and shows complex morphological diversity (Stearn, 1992).

Allium cepa is one of the oldest cultivated vegetables and is currently the second most widely cultivated vegetable in the world after tomato (FAOSTAT, 2018). Other minor *Allium* species, of less economic importance than onion, are grown sporadically in restricted regions only, and were historically of greater importance (Fritsch and Friesen, 2002). The largest producers of shallots and similar minor *Allium* species are China and Japan, with more than 500,000 tons of shallot bulbs produced per year, followed by New Zealand, Mexico, Iran, Iraq, Cambodia, and Cameroon (FAOSTAT, 2018).

In Croatia, minor *Allium* species are cultivated by local farmers and households along the coastal areas of Istria, Kvarner, Dalmatia, and Dalmatian hinterland. They are generally propagated by bulbs and are closely related to common onions. Recently, Puizina (2013) proposed that shallots in Croatia could be divided into three genotypes based on vegetative and generative morphological characteristics: *A. cepa* Aggregatum ($2n = 2x = 16$), *A.* × *proliferum* (Moench) Schard ($2n = 2x = 16$), and *A.* × *cornutum* Clementi ex Vis. ($2n = 3x = 24$), among which *A.* × *cornutum* is the most widespread in the coastal area. Owing to morphological similarities, it is often difficult to distinguish the species in the field, requiring development of fast and reliable methods for discrimination of landraces to support breeding programs or for commercial exploitation.

Onions are rich in antioxidants, mainly quercetin and its glycosides, and are a major source of dietary flavonoids (Slimestad et al., 2007). In addition, flavonoids are responsible for the yellow or red color of onions (Ferioli and D'Antuono, 2016). Although these health-promoting compounds are ubiquitous in onion bulbs, a detailed chemical profile is required for identification, as the content of specific compounds can vary among *Allium* species or cultivars (Griffiths et al., 2002; Slimestad et al., 2007; Ferioli and D'Antuono, 2016).

Domesticated cultivars, local landraces, ecotypes, or wild edible hybrids are gaining interest, from both economic and nutritional standpoints. The basis for agricultural research, breeding programs, and crop improvement is assessment of plant genetic diversity (Fowler and Hodgkin, 2004; Govindaraj et al., 2015). In the recent years, effort is allocated toward identification and characterization of local landraces in order to preserve the genetic structure from erosion as well as to protect local agronomic production systems by means of agricultural, biological and chemical multidisciplinary approach (Jump et al., 2009; Siracusa et al., 2013; Ferioli and D'Antuono, 2016).

Minor *Allium* crops in Croatia belong to three genetically and morphologically different, vegetatively reproduced relatives of the common onion, *A. cepa* L. (Puizina, 2013). Shallots belonging to *A. cepa* Aggregatum are no longer considered to be a different species, but are classified in the common onion group, as *A. cepa* L. species (Fritsch and Friesen, 2002; Rabinowitch and Kamenetsky, 2002; Brickell et al., 2016).

In this study, shallot accessions collected along the Croatian coast and hinterland were evaluated for their morphological properties. Furthermore, chemical composition of these accessions was compared with that of local landraces of common onions and market-available shallot and common onion cultivars. The diversity observed for the tested traits may be useful for preservation of genetic variability in future breeding programs and to protect local agronomic production systems by means of agricultural, biological, and chemical multidisciplinary approach (Jump et al., 2009; Siracusa et al., 2013; Ferioli and D'Antuono, 2016).

MATERIALS AND METHODS

Material

Shallot landraces were collected from 2014 to 2017 across Croatia (**Figure 1**) as part of the National Program of Conservation and Sustainable Use of Plant Genetic Resources. Thirteen shallot landraces were collected along Croatian coastal area, from northern and central Istria, Kvarner, Dalmatia, and Dalmatian hinterland areas. The collected landraces were vegetatively propagated by underground bulbs except IPT023 which was propagated by aerial bulbils.

The field trial was established by the end of October 2016 at the Institute of Agriculture and Tourism in Poreč, Croatia (N 45°13′20.30″, E 13°36′6.49″). The shallot clusters consisted of 2–3 bulbs were planted at distance of 20 cm in row and 30 cm between rows. At least 40 clusters of each accession were planted. In addition to shallot landraces, local landraces of common onion were planted as transplants in the same field at the same time at the same planting density (**Table 1**). Before planting, NPK

FIGURE 1 | Locations of origin of local shallot accessions.

TABLE 1 | Vegetative and generative morphological qualitative descriptors[1] of flowering shallot accessions.

Accession	Foliage color (QL1)	Foliage attitude (QL2)	Leaf diameter (QL3)	Cross-section of leaf (QL4)	Degree of leaf waxiness (QL5)	Shape of mature dry bulbs (QL6)	Presence of bulbils (offsets) (QL7)	Number of bulbils (QL8)	Scape[2] (QL9)	Flower number in umbel (QL10)	Inflorescence[3] (QL11)	Perianth[4] (QL12)	Pistil[5] (QL13)	Stamen morphology[6] (QL14)	Anther color (QL15)	General fertility (QL16)[7]
A.* × *cornutum																
IPT021	Yellow green	Intermediate	Narrow	Square	Weak	Globe	Present	Few (<30)	1	Many (>30)	3	3	2	3	Yellow	Sterile
IPT022	Yellow green	Intermediate	Narrow	Pentagonal	Medium	Broad elliptic	Present	Few (<30)	1	Many (>30)	3	3	2	3	Yellow	Sterile
IPT211	Yellow green	Intermediate	Narrow	Pentagonal	Weak	Ovate (elongated oval)	Present	Few (<30)	1	Many (>30)	3	3	2	3	Yellow	Sterile
IPT212	Yellow green	Intermediate	Narrow	Pentagonal	Medium	Ovate (elongated oval)	Present	Few (<30)	1	Many (>30)	3	3	2	3	Yellow	Sterile
IPT213	Yellow green	Intermediate	Narrow	Pentagonal	Weak	Ovate (elongated oval)	Present	Few (<30)	1	Many (>30)	3	3	2	3	Yellow	Sterile
IPT214	Yellow green	Intermediate	Narrow	Semi-circular	Weak	Ovate (elongated oval)	Present	Few (<30)	1	Many (>30)	3	3	2	3	Yellow	Sterile
A.* × *proliferum																
IPT023	Green	Erect	Medium Broad	Semi-circular	Weak	Ovate (elongated oval)	Present	Few (<30)	2	Many (>30)	2	2	2	2	Green	Sterile
***A. cepa* Aggregatum**																
IPT208	Yellow green	Intermediate	Medium	Concave	Weak	Broad elliptic	Absent	Absent	1	Many (>30)	1	1	1	1	Green	Fertile
IPT217	Yellow green	Intermediate	Narrow	Circular	Weak	Broad oval	Absent	Absent	1	Many (>30)	1	1	1	2	Green	Fertile
IPT218	Yellow green	Intermediate	Narrow	Circular	Weak	Ovate (elongated oval)	Absent	Absent	1	Many (>30)	1	1	1	2	Green	Fertile

[1]Descriptors in table represent qualitative properties observed on shallot accessions (n = 10) with ability to flower, based on ECP/GR descriptors for vegetatively propagated Allium species and the ones described by Puizina (2013). [2]Scape: conic, hollow, simple (1); conic, hollow, caring bulbils in several levels (2). [3]Inflorescence: round, no bulbils (1); prizmatc, carrying bulbils (2); round, carrying bulbils (3). [4]Perianth: star like, green stripe (1); campanulate, green stripe (2); star like, purple stripe (3). [5]Pistil: lower then stamens (1); taller then stamens (2). [6]Stamen morphology: green, A. cepa type (1); green, simple (2); yellow, A. cepa type (3). [7]QL1 to QL16 are labels of the included qualitative descriptors as seen in ***Figure 3****.*

fertilizer (5:20:30) was incorporated in soil at 500 kg ha^{-1} and at begging of March N was applied (urea source) at a rate of 45 kg ha^{-1}. The weeds were removed manually. The plants were grown without irrigation and according to common agricultural practices for onion growing (Lešić et al., 2004). The harvest started at begging of July when at least 50% of pseudo stems bent over for each accession.

Commercial cultivars of common onion were purchased at a local market in July 2017, for comparison of biochemical characteristics with those of the accessions in our collection. The cultivars Redwing (red onion), Legend (yellow onion), and Lang Prince de Bretagne (long bulb shallot) were included in the study.

Morphological Characterization of Local Shallot Landraces

During the vegetative period, the accessions were evaluated according to descriptors for generative organs provided by (Puizina, 2013) and a list of ECP/GR descriptors for vegetatively propagated *Allium* species (IPGRI et al., 2001). Of the 13 coastal shallot accessions collected, only 10 entered reproductive phases, with a flowering period from June 10th to 14th, 2017. These 10 accessions were eligible for morphological differentiation analysis based on flower characteristics. In total, we used 16 qualitative and 10 quantitative plant descriptors for characterization of landraces.

Plants were harvested at maturity and sampled for further analyses after a month of curing in the shade.

Determination of Macro and Micro Elements

Shallot bulbs were dried in an oven with circulating air at 70°C for 48 h, then ground for nutrient analysis. Powdered material (0.5 g) was obtained from each sample, subjected to dry washing in a muffle furnace at 550°C for 5 h, and used to extract P, K, Ca, Mg, Zn, Mn, and Cu after dissolving in 2 mL HCl. P concentration was determined by the vanadate-molybdate yellow color method (Chapman and Pratt, 1961) using a spectrophotometer at 420 nm. K concentrations were measured using flame photometry (Model 410; Sherwood Scientific Ltd., Cambridge, United Kingdom), while Ca, Mg, Zn, Mn, and Cu were determined by atomic absorption spectrometry (Spectraa 220; Varian Inc., Palo Alto, CA, United States). Total N concentration was measured by the micro-Kjeldahl digestion system (Kjeltec system 1026, Foss Inc., Hilleroed, Denmark).

Extraction of Soluble Phenolic Compounds

Extraction of phenolic compounds was performed by ultrasound-assisted extraction in 80% methanol. Briefly, 2 g of sample was homogenized with a rotary bearing mill (Model HOMEX 6, Bioreba AG, Reinach, Switzerland) in 9.5 mL of 80% methanol and 0.5 mL NaCl. The mixture was sonicated for 30 min and left to macerate for 4 h at 20°C. The mixture was filtered and centrifuged at 6000 × *g* for 15 min. The resulting supernatant was collected and diluted to a final volume of 10 mL with extraction solvent. The solution was filtered through a 0.45 μm filter prior to analysis.

Measurement of Total Phenolic Content

Total phenolic content (TPC) was evaluated by the Folin-Ciocalteu assay (Singleton and Rossi, 1965). Sample extracts (0.2 mL) were mixed with 1.4 mL of freshly diluted 0.2 M Folin-Ciocalteu reagent in water. Sodium carbonate (1.4 mL, 6% in distilled water) was added after 1 min and the mixture was vortexed. The reaction mixture was incubated at room temperature and the absorbance of the mixture was read at 750 nm on a UV/Vis spectrophotometer (Model UV-1800, Shimadzu Corporation, Kyoto, Japan). TPC was standardized against gallic acid and expressed as mg of gallic acid equivalents per g sample in fresh weight (FW).

Quantification of Phenolic Compounds

Chromatographic separations were performed by reversed-phase HPLC. The HPLC instrument consisted of a solvent delivery module (Model ProStar 230, Varian Inc., Palo Alto, CA, United States), a column valve module (Model CVM 500, Varian Inc., Palo Alto, CA, United States), UV/Vis detector (Model ProStar 310, Varian Inc., Palo Alto, CA, United States), and a 5 μm RP C18 column (250 mm × 4.6 mm) (Chromsep Omnispher, Varian Inc., Palo Alto, CA, United States). Gradient elution with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) was achieved using the following program: 90% to 25% A, 0 to 55 min; 25% to 2% A, 55 to 57 min; 2% A, 57 to 69 min. Column temperature was held at 30°C, injection volume was 20 μL, and flow rate was 1.0 mL/min. Individual phenolic compounds were identified and quantified using authentic reference standards of quercetin-3,4′-glucoside, quercetin, isoquercetin, chlorogenic, vanillic, and ferulic acids. Quercetin-4′-glucoside was identified using previously published data and quantified by comparing its relative area with the relative area of the isoquercetin (quercetin-3-glucoside) standard.

Determination of Total Antioxidant Capacity

Total antioxidant capacity of various *Allium* accessions was evaluated spectrophotometrically (Model UV-1800, Shimadzu Corporation, Kyoto, Japan) by Ferric Reducing Ability of Plasma (FRAP) (Benzie and Strain, 1996) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays (Brand-Williams et al., 1995). FRAP values were obtained by analyzing a mixture of 1 mL of sample with 2 mL of freshly prepared FRAP reagent at 593 nm after 4 min of reaction time. Results were expressed as mM of Fe^{2+} equivalents per g sample in FW. DPPH radical scavenging activity was determined by analyzing a mixture of 1 mL of the sample with 2 mL of 0.1 mM DPPH radical at 517 nm after 30 min in darkness. The results were expressed as mM of Trolox equivalents per g sample in FW.

Statistical Analysis

The morphological description of flowering accessions was conducted on 10 plants per accession as recommended by the IPGRI et al. (2001). Analysis of macro- and micro-elements, phenolic content, phenolic compounds, and antioxidant capacity were performed in triplicate. Data were analyzed by analysis

of variance (ANOVA) and Partial Least Square (PLS) analysis using Statistica 13.3 (Tibco, Inc). Significant differences were determined at $p \leq 0.05$ and homogenous group means were compared by Tukey's HSD test.

Similarly to Principal Components Regression (PCR), the scope of PLS regression is to form new components that capture most information in the independent variables that is useful for predicting dependent variables, while reducing the dimensionality of the dataset (Garthwaite, 1994). In addition to the information contained in the independent variables, PLS also uses information from dependent variables in the formation of components. As such, PLS is of particular use when there are many independent variables and comparatively little data (Garthwaite, 1994; Helland, 2014). The advantage of PLS regression lies in its exploratory potential. Here the method was applied as an exploratory tool for identification of variables critical in the discrimination between local shallot landraces.

RESULTS

Qualitative and Quantitative Morphological Properties

During the growing season, we observed differences in plant habit (**Figures 2A–C**) and type of inflorescence (**Figures 2D–F**). Therefore, vegetative and generative plant morphological descriptors were used to describe and group the 10 flowering shallot accessions. Morphological plant descriptors are

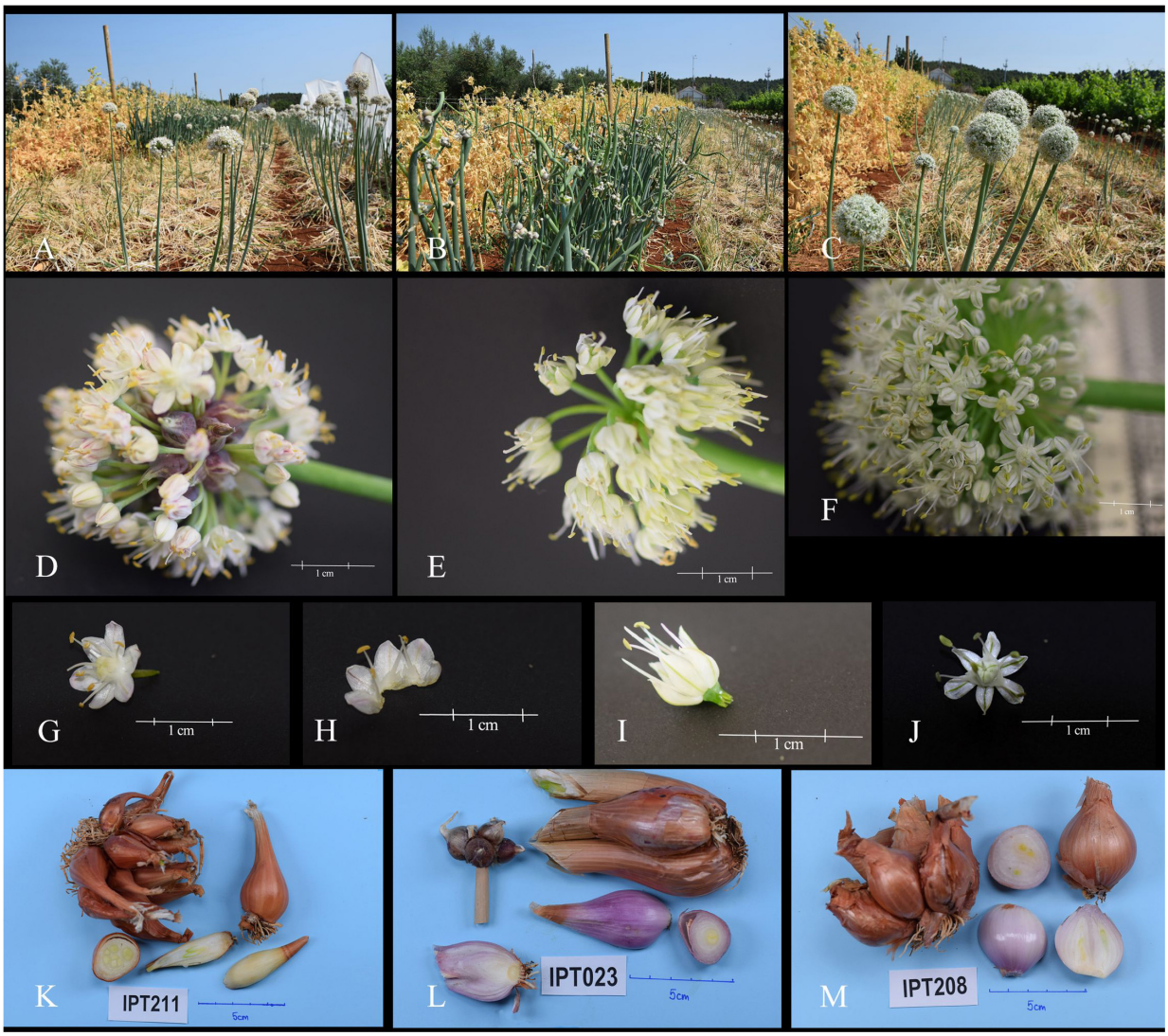

FIGURE 2 | Flowering plant in the field: **(A)** *Allium* × *cornutum*, **(B)** *Allium* × *proliferum*, **(C)** *Allium cepa* Aggregatum. Inflorescence: **(D)** *Allium* × *cornutum*, **(E)** *Allium* × *proliferum*, **(F)** *Allium cepa* Aggregatum. Flower: **(G,H)** *Allium* × *cornutum*, **(I)** *Allium* × *proliferum*, **(J)** *Allium cepa* Aggregatum. Underground bulbs: **(K)** *Allium* × *cornutum*, **(L)** *Allium* × *proliferum*, **(M)** *Allium cepa* Aggregatum.

summarized in **Tables 1**, **2**, and accessions are denoted by their respective species.

The majority of accessions belonged to *A.* × *cornutum* and were characterized by yellow–green foliage color, intermediate foliage attitude, narrow leaf diameter, and the presence of fewer than 30 bulbils in the inflorescence (**Table 1**). Scapes of accessions belonging to the *A.* × *cornutum* group were conic, hollow, simple; round inflorescence with bulbils; perianth purple-green, with pistils taller than stamens; and anthers yellow (**Figures 2D,G**).

A. × *proliferum* accessions were distinguished from *A.* × *cornutum* and *A. cepa* Aggregatumg by green foliage color, erect foliage attitude, medium broad leaf diameter (**Table 1**), prizmic inflorescence, campanulate green striped perianth, and green anther color (**Figures 2E,I**). Scape morphology in *A.* × *proliferum* was gigantic in size, carrying bulbils in several levels (**Figure 2B**), which differed greatly from the other two species.

Allium cepa Aggregatum accessions were characterized by circular to concave leaf cross sections; absence of bulbils in inflorescence; star-like, green striped perianth; pistils lower than stamens; green stamens and anthers; and fertile flowers (**Figures 2F,J** and **Table 1**).

Although bulbs should be the main organ used to differentiate accessions, their shapes were variable and ranged from elongated oval, broad elliptic, globose, and broad oval to broad elliptic in each of the described accessions (**Figures 2K–M** and **Table 1**).

Quantitative morphological characteristics were significantly different among the accessions for all traits studied, except bulb diameter (**Table 2**). Quantitative differences among *Allium* groups were not as clear as qualitative differences. The gigantic nature of *A.* × *proliferum* accession (IPT023) was characterized by greater leaf diameter, cluster mass, scape length, and diameter, whereas the bulb number per cluster was generally smaller (**Table 2**).

Figures 3A,B present PLS analysis of 10 flowering shallot accessions using qualitative and quantitative morphological descriptors presented in **Tables 1**, **2**, respectively. Based on inflorescence (QL11) and perianth (QL12) morphology, all three groups of shallot species could be distinguished from each other (**Figures 2D–J**, **3A**).

A. × *cornutum* shallot accessions could be distinguished from *A. cepa* Aggregatum and *A.* × *proliferum* based on the degree of leaf waxiness (QL5), flower number in umbel (QL10), stamen morphology (QL14), and anther color (QL15), as shown in **Figure 3A**.

The Aggregatum group of shallot accessions could be distinguished from the other two species based on the presence (QL7) and number of bulbils (QL8), shape of mature dry bulbs (QL6), and general fertility (QL16), as seen in **Figures 2F,J,M**, **3A**. Furthermore, *A.* × *proliferum* shallot accessions could be distinguished from *A. cepa* Aggregatum and *A.* × *cornutum* (**Figure 3A**) based on extreme vegetative growth (**Figure 2B**), foliage color (QL1) and attitude (QL2), leaf diameter (QL3) and cross-section shape (QL4), and scape morphology (QL9).

Based on PLS analyses of quantitative descriptors of shallot accessions, it was seen that higher variability separates *A.* × *proliferum* from the other two species in leaf (QN2) and bulb

TABLE 2 | Vegetative and generative morphological quantitative descriptors[1] of flowering shallot accessions.

Accession	Leaf length (cm) (QN1)[2]	Leaf diameter (cm) (QN2)	Number of bulbs per cluster (QN3)	Cluster mass (g/cluster) (QN4)	Bulb diameter (mm) (QN5)	Scape length (cm) (QN6)	Scape diameter (mm) (QN7)	Inflorescence diameter (mm) (QN8)	Flower pedicle length (mm) (QN9)	Stamen length (mm) (QN10)
A. × cornutum										
IPT021	29.60 ± 2.30a[3]	7.56 ± 0.36b	18.40 ± 8.14ab	329.1 ± 48.1bc	34.68 ± 3.93	67.40 ± 1.14bcd	12.79 ± 1.02bcd	45.55 ± 1.66abc	11.58 ± 2.30bc	5.51 ± 0.53c
IPT022	26.50 ± 4.62b	6.70 ± 1.37b	19.00 ± 5.20ab	308.8 ± 88.7bc	33.61 ± 4.69	63.60 ± 5.94bcd	9.68 ± 2.10*cd*	41.99 ± 2.07abc	7.91 ± 0.83de	6.94 ± 0.96abc
IPT211	30.84 ± 5.70ab	7.27 ± 0.90b	32.40 ± 12.46ab	404.4 ± 107.2abc	31.37 ± 2.67	64.60 ± 7.27bcd	9.42 ± 1.93cd	30.50 ± 3.00de	7.09 ± 1.31e	5.57 ± 0.92c
IPT212	26.80 ± 3.56b	7.87 ± 2.00b	33.60 ± 21.8a	310.6 ± 114.5bc	34.45 ± 7.95	65.60 ± 7.09bcd	8.10 ± 1.96d	41.43 ± 4.09abcd	8.06 ± 1.04de	6.71 ± 0.92abc
IPT213	28.30 ± 1.52b	6.36 ± 1.21b	30.80 ± 14.50ab	252.0 ± 34.5c	27.85 ± 1.57	53.70 ± 5.65d	8.38 ± 1.36d	35.90 ± 2.38cde	7.05 ± 0.71e	5.72 ± 0.83c
IPT214	31.10 ± 1.67ab	5.68 ± 1.20b	31.00 ± 19.40ab	295.6 ± 119.2bc	29.52 ± 3.50	59.00 ± 8.83cd	8.57 ± 0.85d	38.27 ± 2.70bcde	7.97 ± 0.57de	6.40 ± 0.42bc
A. × proliferum										
IPT023	38.10 ± 3.97ab	13.00 ± 1.54a	8.00 ± 1.73b	566.7 ± 147.8a	36.38 ± 2.10	103.20 ± 6.42a	22.88 ± 3.19a	27.91 ± 5.27e	10.57 ± 2.53cd	8.32 ± 1.38a
***A. cepa* Aggregatum**										
IPT208	46.64 ± 7.39a	12.86 ± 3.44a	12.20 ± 1.64ab	483.6 ± 54.5ab	36.26 ± 5.58	66.80 ± 18.19bcd	13.17 ± 3.18bcd	52.12 ± 5.42a	16.51 ± 0.45a	7.84 ± 0.54ab
IPT217	28.80 ± 4.56b	6.34 ± 1.57b	17.40 ± 4.72ab	287.9 ± 69.4c	30.96 ± 4.99	73.00 ± 6.20bc	14.15 ± 1.97bc	38.87 ± 4.82bcde	13.88 ± 1.71ab	6.61 ± 0.77abc
IPT218	32.90 ± 4.16ab	6.27 ± 0.48b	12.20 ± 3.70ab	412.0 ± 66.8abc	30.39 ± 13.52	77.40 ± 6.02b	14.85 ± 4.26b	47.73 ± 12.72ab	15.44 ± 2.21a	6.82 ± 0.65abc
p-value	<0.001	<0.001	0.003	<0.001	0,335	<0.001	<0.001	<0.001	<0.001	<0.001

[1]*Descriptors in table represent quantitative morphological properties observed on flowering shallot accessions, based on ECP/GR descriptors for vegetatively propagated Allium species and the ones described by Puizina (2013).* [2]*QN1 to QN10 are labels of the quantitative descriptors as seen in* ***Figure 3****.* [3]*Data are presented as mean ± SD (n = 10). The different letter within column denotes significant difference by Tukey's HSD test at* $p \leq 0.05$.

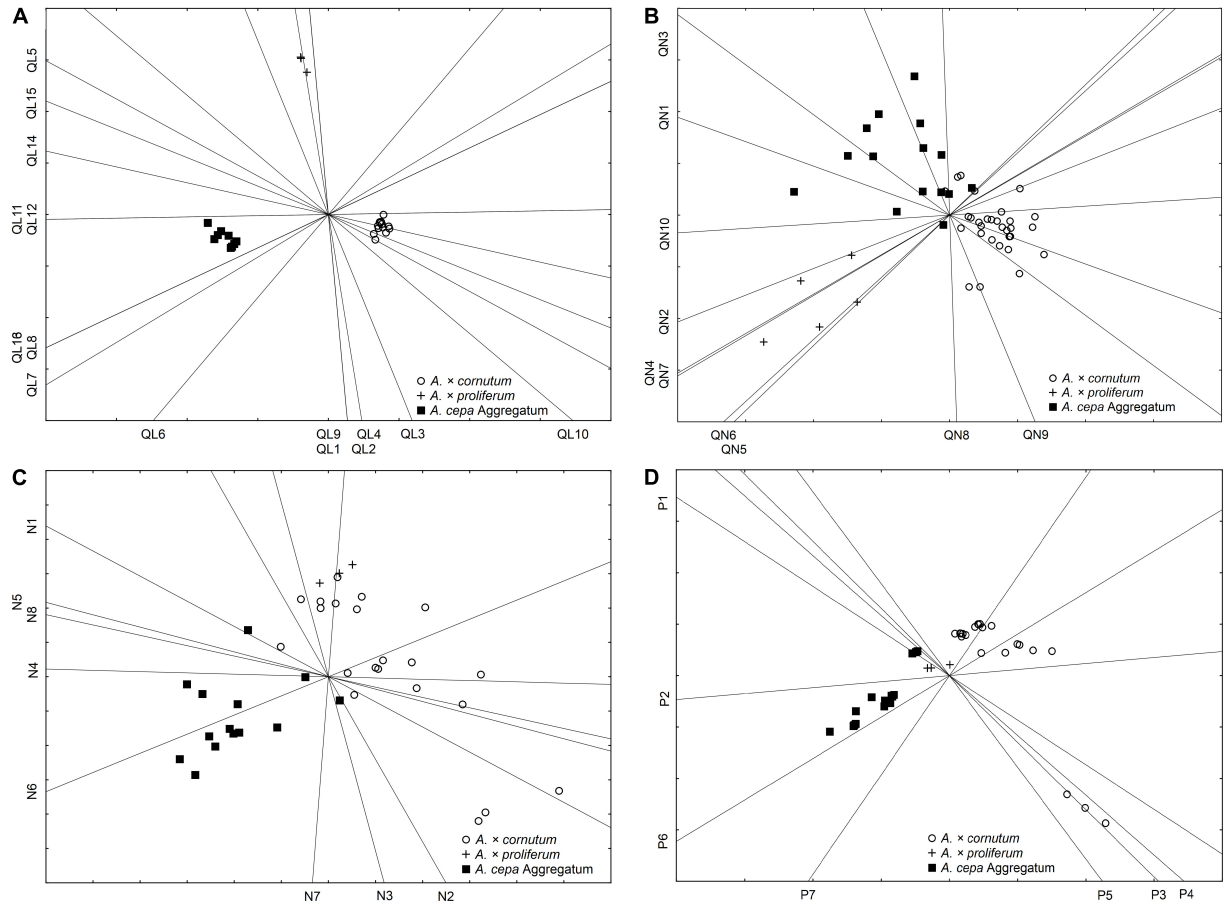

FIGURE 3 | Partial least square (PLS) analysis of local shallot accessions based on **(A)** qualitative morphological descriptors (QL1 to QL16), **(B)** quantitative morphological descriptors (QN1 to QN10), **(C)** nutrient and mineral profiles (N1 to N8), and **(D)** phenolic compounds (P1 to P7).

(QN5) diameter, cluster mass (QN4), and scape length (QN6) and diameter (QN7), confirming gigantism in *A.* × *proliferum* (**Figure 2B**). The number of bulbs in clusters (QN3) and leaf length (QN1) are responsible for most of the variability that differentiated *A.* × *cornutum* from the other two species (**Figures 2A,K**, **3B**). Furthermore, shorter stamen length (QN10) and flower pedicel length (QN9) are the distinguishing factors of *A.* × *cornutum* (**Figure 3B**). Inflorescence diameter (QN8) can be used to distinguish *A. cepa* Aggregatum from *A.* × *cornutum* (**Figure 3B**), particularly in the case of accession IPT208, as seen in **Table 2**.

Nutritional and Mineral Profiles

Mineral profiles of the shallot accessions, commercial onions, and shallot cultivars are shown in **Table 3**. Based on morphological descriptors, the accessions were assigned to different species. Data showed that *A.* × *cornutum* was characterized by significantly higher N, Ca, Mg, and Cu content than those in *A. cepa* Aggregatum, but not P and K content (**Table 3**). The A. × *proliferum* landrace is characterized by significantly lower Mn content than those in *A.* × *cornutum* and *A. cepa* Aggregatum (**Table 3**).

Local shallot accessions had N concentrations from 8.1 ± 0.3 (in IPT022) to 3.7 ± 0.5 g/kg FW (in IPT208), and differed significantly from the values in shallot cultivar Lang Prince (except IPT208) and commercial onion varieties.

The P concentration in commercial shallots did not differ for the majority of shallot accessions, and ranged from 0.5 ± 0.2 g/kg FW (in Lang Prince and IPT214) to more than 0.7 ± 0.1 g/kg FW (in IPT211, IPT216, IPT217, IPT176, and IPT208). Both commercial onion cultivars had much lower P concentrations than that in the local accessions. P concentrations in commercial onions were 2–3 times lower and differed from that in all shallot accessions (local and commercial), where concentrations ranged from 2.34 ± 0.23 g/kg FW (in Lang Prince) to 3.41 ± 0.06 g/kg FW (in IPT022) and 3.36 ± 0.07 g/kg FW (in IPT216). *A. cepa* Aggregatum accession IPT208 had higher Ca concentrations (0.94 ± 0.28 g/kg FW) than that of all accessions belonging to *A.* × *proliferum* and *A. cepa* Aggregatum. On average, Mg concentrations in fresh bulbs of shallot accessions were twofold higher than in commercial onion varieties. Generally, higher Mg was found in IPT022, and commercial onions had the lowest Mg concentrations when compared with all shallot accessions.

TABLE 3 | Mineral content of accessions of local shallot landraces and commercial *Allium* cultivars, expressed on FW.

	N (g/kg FW) (N1)[1]	P (g/kg FW) (N2)	K (g/kg FW) (N3)	Ca (g/kg FW) (N4)	Mg (mg/kg FW) (N5)	Zn (mg/kg FW) (N6)	Mn (mg/kg FW) (N7)	Cu (mg/kg FW) (N8)
Species[2]								
A. × *cornutum*	5.66 ± 1.06a	0.67 ± 0.01	2.79 ± 0.33	0.73 ± 0.12a	215.1 ± 26.8a	5.82 ± 4.36	1.94 ± 0.31a	1.21 ± 0.35a
A. × *proliferum*	4.48 ± 0.11ab	0.56 ± 0.01	2.83 ± 0.12	0.58 ± 0.02ab	195.3 ± 2.1ab	3.82 ± 0.10	1.11 ± 0.03b	1.18 ± 0.10ab
A. cepa Aggregatum	4.85 ± 0.90b	0.72 ± 0.10	2.99 ± 0.26	0.54 ± 0.24b	189.9 ± 14.9b	6.83 ± 4.15	2.10 ± 0.29a	0.95 ± 0.15b
p-value	0.025	0.078	0.143	0.007	0.006	0.489	<0.001	0.030
Accessions								
A.* × *cornutum								
IPT021	5.33 ± 0.09cd[3]	0.63 ± 0.01bc	2.70 ± 0.14cde	0.70 ± 0.11abcde	200.0 ± 15.3cd	3.91 ± 0.08	1.41 ± 0.02def	1.17 ± 0.04bc
IPT022	8.12 ± 0.27a	0.91 ± 0.03a	3.41 ± 0.06a	0.82 ± 0.04abc	261.0 ± 8.7a	6.06 ± 0.07	2.39 ± 0.09bc	1.83 ± 0.19a
IPT211	5.57 ± 0.12bc	0.77 ± 0.04ab	2.94 ± 0.09bc	0.84 ± 0.06ab	236.8 ± 10.1ab	4.18 ± 0.32	1.94 ± 0.02bcde	1.51 ± 0.32ab
IPT212	5.51 ± 0.24c	0.67 ± 0.05bc	2.77 ± 0.06cde	0.75 ± 0.03abcd	213.3 ± 6.8bcd	6.28 ± 3.20	2.09 ± 0.02bcd	1.12 ± 0.23bc
IPT213	5.12 ± 0.19cde	0.61 ± 0.01bc	2.76 ± 0.04cde	0.84 ± 0.01ab	220.3 ± 2.8bc	4.19 ± 0.05	2.21 ± 0.08bc	0.91 ± 0.03c
IPT214	5.05 ± 0.07cde	0.52 ± 0.01cd	2.63 ± 0.06de	0.64 ± 0.01bcdef	189.2 ± 2.1de	12.69 ± 13.34	1.84 ± 0.09bcdef	1.02 ± 0.06c
IPT215	4.93 ± 0.06cde	0.60 ± 0.02bc	2.28 ± 0.06f	0.55 ± 0.05defgh	184.7 ± 3.8de	3.39 ± 0.04	1.72 ± 0.06cdef	0.91 ± 0.10c
A.* × *proliferum								
IPT023	4.48 ± 0.11e	0.56 ± 0.01bc	2.83 ± 0.12bcde	0.58 ± 0.02cdefg	195.5 ± 2.1cde	3.83 ± 0.14	1.11 ± 0.03f	1.18 ± 0.10bc
***A. cepa* Aggregatum**								
IPT176	4.86 ± 0.13cde	0.74 ± 0.10abc	2.61 ± 0.04e	0.37 ± 0.05gh	168.0 ± 5.9e	6.58 ± 4.58	1.79 ± 0.09cdef	0.96 ± 0.04c
IPT208	3.72 ± 0.47f	0.76 ± 0.10ab	2.88 ± 0.03bcd	0.94 ± 0.28a	200.3 ± 20.5cd	3.80 ± 0.04	2.07 ± 0.04bcd	0.80 ± 0.03c
IPT216	6.27 ± 0.06b	0.73 ± 0.07abc	3.36 ± 0.07a	0.41 ± 0.05fgh	197.4 ± 7.7cde	13.28 ± 3.34	1.89 ± 0.03bcde	1.10 ± 0.06bc
IPT217	4.73 ± 0.50de	0.76 ± 0.02ab	3.05 ± 0.05b	0.52 ± 0.08defgh	196.6 ± 0.1cde	3.62 ± 0.01	2.17 ± 0.05bcd	0.82 ± 0.19c
IPT218	4.69 ± 0.43de	0.62 ± 0.15bc	3.04 ± 0.03b	0.46 ± 0.01efgh	185.7 ± 3.5de	7.16 ± 5.17	2.57 ± 0.15ab	1.05 ± 0.06c
Commercial cultivars								
'Redwing'	2.23 ± 0.02 *g*	0.29 ± 0.07e	1.17 ± 0.03 *h*	0.39 ± 0.10 *h*	123.8 ± 11.8f	2.26 ± 0.42	2.23 ± 1.15ef	0.85 ± 0.08c
'Legend'	2.29 ± 0.03 *g*	0.33 ± 0.02de	0.78 ± 0.06 *g*	0.47 ± 0.07efgh	113.5 ± 4.3f	2.61 ± 0.03	3.27 ± 0.02a	0.89 ± 0.10c
Long bulbshallot	3.20 ± 0.13f	0.52 ± 0.18cd	2.34 ± 0.23f	0.70 ± 0.07abcde	192.8 ± 23.2cde	9.88 ± 0.92	1.84 ± 0.09bcdef	1.11 ± 0.23bc
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.211	<0.001	<0.001

[1] *N1–N8 are labels of the included macro- and micro-elements as seen in* ***Figure 3****.*

[2] *The data represent average of accessions belonging to each species.*

[3] *Data are presented as mean ± SD (n = 3). The different letter within column denotes significant difference between accessions of local landraces and commercial cultivars by Tukey's HSD test at* $p \leq 0.05$.

Shallot accessions and commercial samples did not differ significantly in Zn concentrations. The highest Mn in fresh bulbs was found in 'Legend' commercial onions, when compared with all other analyzed samples, except IPT218. The highest concentration of Cu was found in IPT022 (1.83 ± 0.19 g/kg) when compared with all other accessions, except IPT211.

Compared with the cultivars of commercial common onions, local shallot accessions had significantly higher N, P, and K levels, while the content of other minerals was not significantly different (**Table 3**).

The PLS analysis of nutritional and mineral data is shown in **Figure 3C**. *A.* × *proliferum* IPT023 and most *A.* × *cornutum* accessions differed from other groups in P (N2), K (N3), and Mn (N7) content (**Figure 3C**). Ca (N4) and Zn (N6) represented the largest differences between *A. cepa* Aggregatum and *A.* × *cornutum*. Mg (N5) and Cu (N8) levels also contributed to differentiation between *A. cepa* Aggregatum and *A.* × *cornutum*, albeit to a lesser extent owing to comparable levels of these minerals in several shallot accessions from both groups.

Phenolic Profile and Total Antioxidant Capacity

The two most abundant phenolic compounds detected in local shallot accessions were quercetin-4′-glucoside and quercetin-3,4′-diglucoside (**Table 4** and **Figure 4**).

Quercetin-4′-glucoside and quercetin-3,4′-diglucoside concentration in *A.* × *cornutum* ranged from 845.0 ± 100.9 mg/kg FW (in IPT211) to 133.5 ± 31.0 mg/kg FW (in IPT021) and from 213.5 ± 39.2 (in IPT022) to 129.4 ± 1.1 mg/kg FW (in IPT215), respectively (**Table 4**). Quercetin-4′-glucoside and quercetin-3,4′-diglucoside concentration in *A. cepa* Aggregatum ranged from 193.8 ± 22.3 mg/kg FW (in IPT176) to 26.2 ± 7.2 mg/kg FW (in IPT 208) and from 107.0 ± 15.0 mg/kg FW (in

TABLE 4 | Phenolic profiles of accessions local shallot landraces, common onion landraces, and commercial *Allium* cultivars expressed in mg/kg FW.

	Quercetin-4′-glucoside (P1)[1]	Quercetin-3,4′-diglucoside (P2)	Quercetin (P3)	Chlorogenic acid (P4)	Isoquercetin (P5)	Vanillic acid (P6)	Ferulic acid (P7)
***Species*[2]**							
A. × *cornutum*	337.4 ± 256.5a[3]	168.8 ± 35.8a	26.2 ± 11.7	30.7 ± 5.3	20.8 ± 3.5	n.d.[4]	14.6 ± 0.7
A. × *proliferum*	213.2 ± 52.7ab	124.4 ± 30.8ab	n.d.	n.d.	n.d.	n.d.	14.4 ± 1.5
A. cepa Aggregatum	109.3 ± 63.8b	77.7 ± 31.9b	n.d.	n.d.	n.d.	11.1 ± 4.2	19.4 ± 5.5
p-value	0.006	<0.001	–	–	–	–	0.151
Accessions							
A.* × *cornutum							
IPT021	133.5 ± 31.0efgh	140.1 ± 28.1de	n.d.	n.d.	n.d.	n.d.	n.d.
IPT022	547.1 ± 104.5b	213.5 ± 39.2a	15.8 ± 3.0b	n.d.	n.d.	n.d.	n.d.
IPT211	845.0 ± 100.9a	191.7 ± 19.3abc	36.6 ± 2.7a	34.5 ± 1.8a	20.8 ± 3.5	n.d.	14.6 ± 0.7c
IPT212	174.8 ± 3.0efg	169.8 ± 6.8abcd	n.d.	n.d.	n.d.	n.d.	n.d.
IPT213	214.5 ± 25.1de	142.6 ± 25.9cde	n.d.	n.d.	n.d.	n.d.	n.d.
IPT214	301.8 ± 13.7cd	194.1 ± 5.8ab	n.d.	25.1 ± 0.3b	n.d.	n.d.	n.d.
IPT215	145.4 ± 22.7efgh	129.4 ± 1.1def	n.d.	n.d.	n.d.	n.d.	n.d.
A.* × *proliferum							
IPT023	213.2 ± 52.7de	124.4 ± 30.8def	n.d.	n.d.	n.d.	n.d.	14.4 ± 1.5c
***A. cepa* Aggregatum**							
IPT176	193.8 ± 22.3def	107.0 ± 15.0efg	n.d.	n.d.	n.d.	10.7 ± 0.9	17.7 ± 1.6b
IPT208	26.2 ± 7.2 *h*	27.0 ± 5.8hi	n.d.	n.d.	n.d.	n.d.	n.d.
IPT216	67.8 ± 11.4fgh	64.3 ± 11.3gh	n.d.	n.d.	n.d.	14.5 ± 2.5	14.4 ± 0.8c
IPT217	160.5 ± 6.0efg	106.9 ± 3.8efg	n.d.	n.d.	n.d.	8.4 ± 0.4	17.3 ± 0.2b
IPT218	98.2 ± 0.5efgh	83.1 ± 1.5fg	n.d.	n.d.	n.d.	10.0 ± 0.3	28.2 ± 0.3a
A. cepa							
IPT003	21.2 ± 5.4 *h*	10.0 ± 3.4i	n.d.	n.d.	n.d.	n.d.	n.d.
IPT004	29.6 ± 12.8 *h*	24.3 ± 5.6hi	n.d.	n.d.	n.d.	n.d.	n.d.
Commercial cultivars							
'Redwing'	57.2 ± 4.5gh	30.1 ± 2.2hi	n.d.	n.d.	n.d.	n.d.	n.d.
'Legend'	222.4 ± 20.1de	121.2 ± 11.5def	n.d.	n.d.	n.d.	n.d.	18.9 ± 0.9b
Long bulbshallot	380.4 ± 58.4c	146.9 ± 13.0bcde	n.d.	n.d.	n.d.	n.d.	n.d.
p-value	<0.001	<0.001	<0.001	0.006	–	0.177	<0.001

[1] *P1–P7 are labels of phenolic compounds as seen in* ***Figure 3****.*
[2] *The data represent average of accessions belonging to each species.*
[3] *Data are presented as mean ± SD (n = 3). The different letter within column denotes significant difference between accessions of local landraces and commercial cultivars by Tukey's HSD test at p ≤ 0.05.*
[4] *n.d., not determined.*

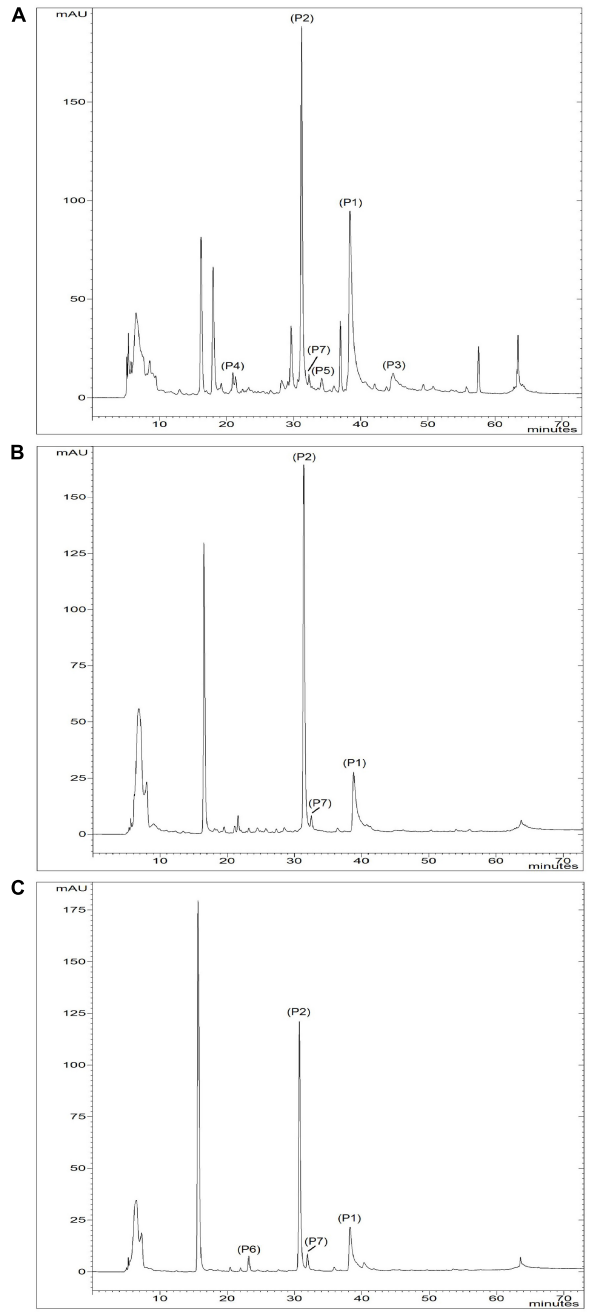

FIGURE 4 | Chromatograms of **(A)** *A.* × *cornutum* IPT211, **(B)** *A.* × *proliferum* IPT023, and **(C)** *A. cepa* Aggregatum IPT176 at 280 nm. Peaks: (P1) quercetin-4′-glucoside; (P2) quercetin-3,4′-glucoside; (P3) quercetin; (P4) Chlorogenic acid; (P5) Isoquercetin; (P6) Vanillic acid; (P7) Ferulic acid.

IPT176) to 27.0 ± 5.8 mg/kg FW (in IPT208), respectively (**Table 4**). Quercetin-4′-glucoside and quercetin-3,4′-diglucoside concentration in *A.* × *proliferum* (IPT023) were 213.2 ± 52.7 mg/kg FW and 124.4 ± 30.8 mg/kg FW, respectively, which lie between the quercetin-4′-glucoside levels measured in *A.* × *cornutum* and *A. cepa* Aggregatum (**Table 4**).

Quercetin was detected in *A.* × *cornutum* IPT211 and IPT022 and chlorogenic acid was detected in *A.* × *cornutum* IPT211 and IPT214 (**Table 4**). Ferulic acid was detected in *A.* × *cornutum* IPT211 and *A. cepa* Aggregatum IPT176, IPT216, IPT217, and IPT218 (**Table 4**).

Vanillic acid (P6) was detected in *A. cepa* Aggregatum IPT176, IPT216, IPT217, and IPT218 (**Table 4**). In addition, isoquercetin was detected in *A.* × *cornutum* IPT211, which contained the most abundant and diverse phenolic compound profile of all tested accessions and cultivars (**Figure 4** and **Table 4**).

Local common onion landraces (IPT003 and IPT004) and the 'Redwing' commercial common onion had levels of quercetin-4′-glucoside and quercetin-3,4′-diglucoside comparable to those in the accessions of *A. cepa* Aggregatum, except IPT176 (**Table 4**).

Commercial 'Legend' yellow common onions and commercial 'Lang prince de Bretagne' long bulb shallots had levels of quercetin-3,4′-diglucoside comparable to those in *A.* × *proliferum* IPT023, several *A.* × *cornutum* accessions (IPT021, IPT213, and IPT215), and several *A. cepa* Aggregatum (IPT176 and IPT217) (**Table 4**).

Average species antioxidant capacities were comparable between *A.* × *cornutum* accessions and *A.* × *proliferum,* but were significantly lower in *A. cepa* Aggregatum (**Table 5**). *A.* × *cornutum* IPT211 and IPT022 had the highest FRAP and DPPH quenching levels, while the lowest values were measured in local common onion varieties (IPT003 and IPT004) (**Table 5**).

Total phenolic content results reflect phenolic profiles of local accessions (**Table 5**). In *A.* × *cornutum,* TPC ranged from 1.96 ± 0.01 mg GAE/g FW (in IPT211) to 0.99 ± 0.02 mg GAE/g FW (in IPT021) (**Table 5**). In *A. cepa* Aggregatum, TPC levels ranged from 0.93 ± 0.03 mg GAE/g FW (in IPT216) to 0.71 ± 0.02 mg GAE/g FW (in IPT218) (**Table 5**). In *A.* × *proliferum,* IPT023 TPC was 1.28 ± 0.04 mg GAE/g FW, which was comparable to TPC levels in *A.* × *cornutum* (**Table 5**). *A. cepa* Aggregatum had significantly lower TPC levels than that in *A.* × *cornutum* and *A.* × *proliferum* (**Table 5**). The commercial shallot cultivar and commercial yellow onion 'Legend' had TPC levels similar to that in *A.* × *cornutum* accessions; while the commercial red onion cultivar 'Redwing' had TPC levels comparable to *A. cepa* Aggregatum accessions (**Table 5**). Local common onion varieties (IPT003 and IPT004) had the lowest TPC values (**Table 5**).

Among local shallot accessions, *A.* × *cornutum* IPT211 had the most abundant and diverse phenolic compound profile (**Figure 4** and **Table 4**). IPT211 also demonstrated high antioxidant capacity, as shown by FRAP and radical scavenging ability, making it the most interesting accession for further studies (**Table 5**).

Phenolic profile data was processed by PLS analysis to further examine the differences between local shallot accessions (**Figure 3D**). The property responsible for the most variability was quercetin-4′-glucoside (P1) content, followed by quercetin-3,4′-diglucoside (P2) content (**Figure 3D**).

Based on quercetin-3,4′-diglucoside (P2) levels, local shallot accessions can be divided into three groups, *A.* × *cornutum*, *A.* × *proliferum,* and *A. cepa* Aggregatum (**Figure 3D**).

TABLE 5 | Antioxidant capacity and total phenolic content in local shallot landraces, local common onion landraces, and commercial *Allium* cultivars.

	DPPH assay – mM TEQ/ FW	FRAP assay – mM Fe2+EQ/ g FW	TPC – mg GAEQ/ g FW
***Species*[1]**			
A. × *cornutum*	1.20 ± 0.34a	2.53 ± 1.46a	1.20 ± 0.37a
A. × *proliferum*	1.23 ± 0.09a	2.09 ± 0.01ab	1.28 ± 0.04a
A. cepa Aggregatum	0.73 ± 0.07b	1.34 ± 0.13b	0.80 ± 0.09b
p-value	<0.001	0.014	<0.001
Accession			
A.* × *cornutum			
IPT021	0.84 ± 0.01fg2	1.50 ± 0.02h	0.99 ± 0.02ef
IPT022	1.62 ± 0.01b	3.31 ± 0.04b	1.47 ± 0.06b
IPT211	1.74 ± 0.01a	5.73 ± 0.08a	1.96 ± 0.01a
IPT212	0.81 ± 0.01fgh	1.54 ± 0.01gh	0.85 ± 0.04ghi
IPT213	1.24 ± 0.02c	1.96 ± 0.01e	1.00 ± 0.05ef
IPT214	1.07 ± 0.03de	1.86 ± 0.03ef	1.14 ± 0.04d
IPT215	1.05 ± 0.01e	1.81 ± 0.02f	1.00 ± 0.04e
A.* × *proliferum			
IPT023	1.23 ± 0.09c	2.09 ± 0.01d	1.28 ± 0.04c
***A. cepa* Aggregatum**			
IPT176	0.77 ± 0.09fgh	1.13 ± 0.06i	0.80 ± 0.02ijk
IPT208	0.64 ± 0.02i	1.23 ± 0.01i	0.81 ± 0.01hij
IPT216	0.81 ± 0.04fgh	1.43 ± 0.07h	0.93 ± 0.03efg
IPT217	0.74 ± 0.02ghi	1.44 ± 0.01h	0.73 ± 0.02jkl
IPT218	0.72 ± 0.02hi	1.42 ± 0.02h	0.71 ± 0.02kl
A. cepa			
IPT003	0.42 ± 0.01k	1.15 ± 0.03i	0.36 ± 0.01n
IPT004	0.53 ± 0.01j	1.27 ± 0.03i	0.46 ± 0.02m
Commercial cultivars			
'Redwing'	0.76 ± 0.02gh	1.66 ± 0.02g	0.67 ± 0.01l
'Legend'	0.89 ± 0.01f	2.09 ± 0.07d	0.91 ± 0.02fgh
Long bulbshallot	1.18 ± 0.05cd	2.40 ± 0.08c	1.11 ± 0.02d
p-value	<0.001	<0.001	<0.001

[1]The data represent average of accessions belonging to each species.
[2]Data are presented as mean ± SD (n = 3). The different letter within column denotes significant difference between accessions of local landraces and commercial cultivars by Tukey's HSD test at p ≤ 0.05.

Furthermore, *A. cepa* Aggregatum accessions were distinguished from other groups by the presence of vanillic acid (P6), with the exception of IPT208 (**Figure 3D** and **Table 4**). *A.* × *cornutum* had the most variable phenolic profile, as seen with quercetin-4′-glucoside (P1), quercetin (P3), chlorogenic acid (P4), and isoquercetin (P5) content (**Figure 3D**). These results indicate that local shallot accessions can be discriminated based on their phenolic profiles.

DISCUSSION

Qualitative and Quantitative Morphological Properties

In this study, based on morphological observations of reproductive and vegetative plant traits, the accessions belonging to *A. cepa* Aggregatum, ($2n = 2x = 16$), *A.* × *proliferum* Moench Schrad. ($2n = 2x = 16$), and *A.* × *cornutum* Clementi ex Vis. ($2n = 3x = 24$) were characterized. Among the analyzed accessions characterized using EC/PGR plant morphological descriptors, six belong to *A.* × *cornutum* (IPT021, IPT022, IPT211, IPT212, IPT213, IPT214, and IPT215), one to *A.* × *proliferum* (IPT023), and three to *A. cepa* Aggregatum (IPT217, IPT281, and IPT208).

The *A.* × *cornutum* group is particularly interesting, since it is grown in a relatively narrow coastal region and on islands in Croatia, and has two main common names. In the southern part of the coast (Dalmatia) it is known as 'Ljutika,' while in the northern part (Istria) it is known as 'Škalonja.' A genetically similar species named 'Pran,' can be found in India (Fredotović et al., 2017). Complexity of the triparental origin of allotriploid *A.* × *cornutum* was previously studied by Friesen and Klaas (1998). However, Puizina et al. (1999) found evidence that two of three parents of triploid viviparous *A.* × *cornutum* were *A. cepa* and *A. roylei*. Combined molecular phylogenic and cytogenetic studies by Fredotović et al. (2014) provided evidence that the third putative parent of *A.* × *cornutum* was the wild Asian species *A. pskemense* B. Fedtsch.

Unlike *A.* × *cornutum*, *A.* × *proliferum* is only occasionally found in home gardens. It is a spontaneous hybrid between *A. cepa* and *A. fistulosum* L., and is commonly known as tree onion or Egyptian onion (Puizina and Papeš, 1996; Maass, 1997; Friesen and Klaas, 1998). It is characterized by underdeveloped underground bulbs and very wide diameter of scape, which bares several levels of sprouting bulbils and ends with prizmatic inflorescence.

The shallots of *A. cepa* Aggregatum are more important in the continental region of Croatia (**Figure 1**, IPT216) (personal observation), and their morphological diversity will be the subject of a future study. These "onion-like" shallots are cultivated around the world, including in Europe, and the same species are known by different common names. In addition, the same common name is sometimes used for different species. Therefore, simple and fast tools for evaluation at the phenological level to provide quick classification on-site for breeders or for curators of genetic banks is potentially very useful.

Partial least square analysis of qualitative and quantitative plant morphological descriptors used in this study confirmed the importance of several qualitative traits for accession characterization (**Figures 3A,B**). The accessions in this study were clearly separated by inflorescence and perianth morphology. To distinguish *A.* × *cornutum* from *A.* × *proliferum,* and *A. cepa* Aggregatum, the degree of leaf waxiness, flower number in umbel, stamen morphology, and anther color were the most important morphological descriptors. *A.* × *proliferum* was distinguished from the other groups by several qualitative traits, such as foliage color and attitude, leaf diameter and cross-section shape, and scape morphology. In contrast, based on *A.* × *proliferum* gigantism, quantitative descriptors, such as leaf and bulb diameter, cluster mass, scape length, and diameter might also be employed for discrimination among shallots (**Figures 3A,B**). *A. cepa* Aggregatum is characterized by lack of bulbils in inflorescence, shape of dry bulb, and fertility, when compared with *A.* × *cornutum* and *A.* × *proliferum*. Although

the ECP/GR descriptor list is very comprehensive, in the case of a large number of accessions, the shorter list may be utilized for discriminating accessions.

Nutritional and Mineral Profiles

The main minerals found were N, K, and Ca, while P and Zn were also detected at considerable levels. The mineral content of shallots in our study was similar to values suggested by USDA (2018) for raw shallots, although Ca levels were approximately twofold higher than those reported.

The differences in mineral composition among genotypes and species in our study did not result from differences in cultivation practices or environmental factors, as was suggested for onions and garlic by Ariyama et al. (2006), Petropoulos et al. (2015, 2018), and Vadalà et al. (2016). As the shallot plants were grown in the same field (same soil type and farming practices), the results of our study were because of genotypic differences. The observed differences in mineral content among genotypes may be related to mechanisms controlling nutrient uptake, translocation, or utilization. Our results suggest that commercial common onion cultivars are generally less efficient in nutrient metabolism than local shallot accessions.

The content of phenolic compounds in plant tissues are often negatively affected by high N-nutrition (Treutter, 2010), although experiments with onions showed no significant difference in quercetin-4′-glucoside content between unfertilized onions and onions that received nitrogen fertilizers (Mogren et al., 2007). It is interesting that our accession IPT022 had the highest N concentration in fresh tissue and is among the landraces with higher concentrations of main phenolic compounds. Therefore, it seems that genotype is not related to N-metabolism efficiency or phenolic compound accumulation.

Phenolic Profile and Total Antioxidant Capacity

The activities of phenolic acids and flavonoids as antioxidants are directly connected to their ability to reduce oxidizing agents, such as free radicals, via functional hydroxyl groups (Wright et al., 2001). Flavonoids are usually present in plants in glycosylated form, resulting in reduced radical scavenging activity, but increased water solubility (Rice-Evans et al., 1997).

As previously reported, the two major phenolic compounds in onion varieties are quercetin-4′-glucoside and quercetin-3,4′-diglucoside (Yang et al., 2004; Bonaccorsi et al., 2005, 2008; Beretta et al., 2017; Fredotović et al., 2017). Soininen et al. (2014) found that long bulb shallot varieties have higher levels of both compounds than round bulb shallot varieties. In our study the content of phenolic compounds was not directly related to bulb shape, since all variety of shapes were found regardless of species. However, when the values for quercetin-4′-glucoside and quercetin-3,4′-diglucoside were averaged for all accessions belonging to same species, we found significantly higher content in *A.* × *cornutum* and *A.* × *proliferum* than in *A. cepa* Aggregatum.

Several minor compounds were detected in some of the investigated accessions, which helped in their differentiation (**Figure 3D**). Vanillic and ferulic acids were detected in all investigated *A. cepa* Aggregatum accessions, except in IPT208, which has stamen morphology of *A. cepa* type indicating close genetic similarity. Ferulic acid was also detected in *A.* × *cornutum* IPT211 and *A.* × *proliferum* IPT023. Beretta et al. (2017) reported the presence of coumaric and ferulic acids in common onions, and ferulic acid in bunching onions, but not in shallots. Prakash et al. (2007) reported the presence of gallic, ferulic, and protocatechuic acids in four varieties of *A. cepa*. Vanillic, caffeic, ferulic, and chlorogenic acids were detected in addition to the main flavonoids in fresh cut onions, as demonstrated by Chen et al. (2016). In the analyzed samples, chlorogenic acid was detected only in *A.* × *cornutum* IPT211 and IPT214. The ability to identify and characterize local landraces by phenolic profile has been reported previously in different species, cultivars (Riggi et al., 2013; Lo Bianco et al., 2017), and local landraces of *A. cepa* (Riggi et al., 2013). In our study, the phenolic profile proved to be a powerful tool to discriminate among local shallot accession groups, especially with inclusion of minor phenolic compounds. High levels of the main flavonols, as well as great diversity in minor phenolic compounds suggest *A.* × *cornutum* IPT211 accession as a prime candidate for further agronomic, genetic, and biochemical studies.

Total phenolic content was determined by the colorimetric Folin–Ciocalteu method, which measures oxidation of phenolic compounds, and the results should correlate well with the estimated antioxidant capacity (Prior et al., 2005). The estimated antioxidant capacity of biological systems should be evaluated using at least two methods to account for interfering compounds (Schlesier et al., 2002; Ozgen et al., 2006). In our study two methods, DPPH free radical quenching and FRAP, were selected. Each rely on electron transfer to determine antioxidant capacity (Prior et al., 2005). In agreement with our study, shallot cultivars commonly have higher levels of flavonoids, TPC, and antioxidant capacity compared with common onion (Yang et al., 2004; Lu et al., 2011; Beretta et al., 2017). Additionally, our results showed that TPC and antioxidant capacity also differ among shallot species found in Croatia, especially *A.* × *cornutum* and *A. cepa* Aggregatum accessions. It is known that TPC and antioxidant activity in plants change with growing conditions (Heimler et al., 2017), and compound extraction methods (Alothman et al., 2009). A study conducted by Pan et al. (2018) showed that quercetin glucosides in *Allium* species, especially quercetin-4′-glucoside, have great potential as tumor cell growth inhibitors. Higher TPC values in *Allium* methanolic extracts correlated with higher *in vitro* radical scavenging ability and stronger inhibition of tumor cell proliferation (Fredotović et al., 2017). Similarly, in our study, shallot accessions or commercial cultivars with higher TPC exhibited stronger *in vitro* antioxidative effects.

Local landraces are of paramount importance for local agro-economic systems. By providing detailed morphological and chemical characteristics, these landraces can be appropriately preserved and evaluated in addition to the commercial varieties. In this study, shallot accessions important in coastal Croatia were characterized and compared with commercial *Allium*

varieties. Local accessions in our study were differentiated according to inflorescence and perianth morphology. The most important morphological descriptors that separated *A.* × *cornutum* from *A.* × *proliferum* and *A. cepa* Aggregatum were degree of leaf waxiness, flower number in umbel, stamen morphology, and anther color. *A.* × *cornutum* and *A.* × *proliferum* exhibited higher antioxidative capacity and total phenolic content compared with *A. cepa* Aggregatum. *A.* × *cornutum* is characterized by significantly higher N, Ca, Mg, and Cu content compared with *A. cepa* Aggregatum, while *A.* × *proliferum* is characterized by significantly lower Mn content. Our results suggest that the investigated landraces possess excellent nutritional qualities, which rival, or even exceed, the quality of commercially developed varieties, especially in terms of the diversity of minor phenolic compounds. The *A.* × *cornutum* accession IPT211 was found to be of particular interest because of its biochemical wealth and diversity. However, further studies are needed to characterize bioactive constituents in greater depth, which may unravel the benefits and potential new applications of the rediscovered local landraces.

AUTHOR CONTRIBUTIONS

DB and SGB designed the experiments. SGB, JP, BU, GD, and NM executed the experiments and analyzed the results. DB, SGB, JP, BU, GD, and NM discussed the results and conclusion of the study. SGB, JP, BU, and NM wrote the manuscript. DB and SGB edited manuscript drafts. All authors approved the manuscript.

FUNDING

This research has been supported by the project KK.01.1.1.01.0005 Biodiversity and Molecular Plant Breeding, Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), Zagreb, Croatia.

REFERENCES

Alothman, M., Bhat, R., and Karim, A. A. (2009). Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chem.* 115, 785–788. doi: 10.1016/j.foodchem.2008.12.005

Ansari, N. A. (2007). Onion cultivation and production in Iran. *Middle East. Russ. J. Plant Sci. Biotechnol.* 1, 26–38.

Ariyama, K., Nishida, T., Noda, T., Kadokura, M., and Yasui, A. (2006). Effects of fertilization, crop year, variety, and provenance factors on mineral concentrations in onions. *J. Agric. Food Chem.* 54, 3341–3350. doi: 10.1021/JF0525481

Benzie, I. F. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* 239, 70–76. doi: 10.1006/abio.1996.0292

Beretta, H. V., Bannoud, F., Insani, M., Berli, F., Hirschegger, P., Galmarini, C. R., et al. (2017). Relationships among bioactive compounds content and the antiplatelet and antioxidant activities of six allium vegetable species. *Food Technol. Biotechnol.* 55, 266–275. doi: 10.17113/ftb.55.02.17.4722

Block, E. (2010). *Garlic and Other Alliums?: The Lore and The Science*, 1st Edn. London: Royal Society of Chemistry.

Bonaccorsi, P., Caristi, C., Gargiulli, C., and Leuzzi, U. (2005). Flavonol glucoside profile of Southern Italian red onion (Allium cepa L.). *J. Agric. Food Chem.* 53, 2733–2740. doi: 10.1021/jf048152r

Bonaccorsi, P., Caristi, C., Gargiulli, C., and Leuzzi, U. (2008). Flavonol glucosides in *Allium* species: a comparative study by means of HPLC–DAD–ESI-MS–MS. *Food Chem.* 107, 1668–1673. doi: 10.1016/j.foodchem.2007.09.053

Brand-Williams, W., Cuvelier, M. E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci. Technol.* 28, 25–30. doi: 10.1016/S0023-6438(95)80008-5

Brickell, C. D., Alexander, C., Cubey, J. J., David, J. C., Hoffman, M. H. A., Malécot, V., et al. (2016). *International Code of Nomenclature for Cultivated Plants (ICNCP or Cultivated Plant Code)*, 9 th Edn. Leuven: International Society for Horticultural Science.

Chapman, H. D., and Pratt, P. F. (1961). *Methods of Analysis for Soils, Plants and Waters*, 1st Edn. Berkley, MI: Press.

Chen, C., Hu, W., Zhang, R., Jiang, A., and Zou, Y. (2016). Levels of phenolic compounds, antioxidant capacity, and microbial counts of fresh-cut onions after treatment with a combination of nisin and citric acid. *Hortic. Environ. Biotechnol.* 57, 266–273. doi: 10.1007/s13580-016-0032-x

Cumo, C. (2015). *Foods That Changed History?: How Foods Shaped Civilization From the Ancient World to THE Present*. Santa Barbara, CA: ABC-CLIO.

FAOSTAT (2018). *Food and Agriculture Organization Corporate Statistical Database*. Available at: http://www.fao.org/faostat/en/#data/QC/visualize [Accessed May 4, 2018].

Ferioli, F., and D'Antuono, L. F. (2016). Evaluation of phenolics and cysteine sulfoxides in local onion and shallot germplasm from Italy and Ukraine. *Genet. Resour. Crop Evol.* 63, 601–614. doi: 10.1007/s10722-015-0270-2

Fowler, C., and Hodgkin, T. (2004). Plant genetic resources for food and agriculture: assessing global availability. *Annu. Rev. Environ. Resour.* 29, 143–179. doi: 10.1146/annurev.energy.29.062403.102203

Fredotović, Ž, Šamanić, I., Weiss-Schneeweiss, H., Kamenjarin, J., Jang, T.-S., and Puizina, J. (2014). Triparental origin of triploid onion, *Allium*? × ?*cornutum* (Clementi ex Visiani, 1842), as evidenced by molecular, phylogenetic and cytogenetic analyses. *BMC Plant Biol.* 14:24. doi: 10.1186/1471-2229-14-24

Fredotović, Ž, Šprung, M., Soldo, B., Ljubenkov, I., Budić-Leto, I., Bilušić, T., et al. (2017). Chemical composition and biological activity of allium cepa L. and *Allium* × *cornutum* (Clementi ex Visiani 1842) methanolic extracts. *Molecules* 22:448. doi: 10.3390/molecules22030448

Friesen, N., and Klaas, M. (1998). Origin of some minor vegetatively propagated allium crops studied with RAPD and GISH. *Genet. Resour. Crop Evol.* 45, 511–523. doi: 10.1023/A:1008647700251

Fritsch, R. M., and Friesen, N. (2002). "Evolution, domestication and taxonomy," in *Allium Crop Science: Recent Advances*, eds H. D. Rabinowitch and L. Currah (Wallingford: CABI), 5–30. doi: 10.1079/9780851995106.0005

Garthwaite, P. H. (1994). An interpretation of partial least squares. *J. Am. Stat. Assoc.* 89, 122–127. doi: 10.2307/2291207

Govindaraj, M., Vetriventhan, M., and Srinivasan, M. (2015). Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. *Genet. Res. Int.* 2015, 1–14. doi: 10.1155/2015/431487

Griffiths, G., Trueman, L., Crowther, T., Thomas, B., and Smith, B. (2002). Onions - a global benefit to health. *Phyther. Res.* 16, 603–615. doi: 10.1002/ptr.1222

Heimler, D., Romani, A., and Ieri, F. (2017). Plant polyphenol content, soil fertilization and agricultural management: a review. *Eur. Food Res. Technol.* 243, 1107–1115. doi: 10.1007/s00217-016-2826-6

Helland, I. (2014). "Partial least squares regression," in *Encyclopedia of Statistical Sciences*, ed. N. Balakrishnan (Chichester: John Wiley & Sons, Ltd.), doi: 10.1002/9781118445112.stat03287

IPGRI, ECP/Gr, and AVRDC (2001). *Descriptors for Allium (Allium spp.) International Plant Genetic Resources Institute,Rome,Italy*. Taipei: Asian Vegetable Research and Development Center.

Jump, A. S., Marchant, R., and Peñuelas, J. (2009). Environmental change and the option value of genetic diversity. *Trends Plant Sci.* 14, 51–58. doi: 10.1016/j.tplants.2008.10.002

Lešić, R., Borošić, J., Buturac, I., Ćustić, M. H., Poljak, M., and Romić, D. (2004). *Povrcìarstvo. Zrinski*. Available at: http://bib.irb.hr/prikazi-rad?rad=183962 [Accessed August 10, 2017].

Lo Bianco, M., Siracusa, L., Dattilo, S., Venora, G., and Ruberto, G. (2017). Phenolic fingerprint of sicilian modern cultivars and durum wheat landraces: a tool to assess biodiversity. *Cereal Chem. J.* 94, 1045–1051. doi: 10.1094/CCHEM-06-17-0125-R

Lu, X., Wang, J., Al-Qadiri, H. M., Ross, C. F., Powers, J. R., Tang, J., et al. (2011). Determination of total phenolic content and antioxidant capacity of onion (*Allium cepa*) and shallot (*Allium oschaninii*) using infrared spectroscopy. *Food Chem.* 129, 637–644. doi: 10.1016/j.foodchem.2011.04.105

Maass, H. I. (1997). Genetic diversity in the top onion, *Allium* × *proliferum* (Alliaceae), analysed by isozymes. *Plant Syst. Evol.* 208, 35–44. doi: 10.2307/23643317

Mogren, L. M., Olsson, M. E., and Gertsson, U. E. (2007). Effects of cultivar, lifting time and nitrogen fertiliser level on quercetin content in onion (*Allium cepa* L.) at lifting. *J. Sci. Food Agric.* 87, 470–476. doi: 10.1002/jsfa.2735

Ohri, D., Fritsch, R. M., and Hanelt, P. (1998). Evolution of genome size in allium (Alliaceae). *Plant Syst. Evol.* 210, 57–86. doi: 10.1007/BF00984728

Ozgen, M., Reese, R. N., Tulio, A. Z. Jr., Scheerens, J. C., and Miller, A. R. (2006). Modified 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) methods. *J. Agric. Food Chem.* 54, 1151–1157. doi: 10.1021/JF051960D

Pan, Y., Zheng, Y. M., and Ho, W. S. (2018). Effect of quercetin glucosides from allium extracts on HepG2, PC-3 and HT-29 cancer cell lines. *Oncol. Lett.* 15, 4657–4661. doi: 10.3892/ol.2018.7893

Petropoulos, S., Fernandes, Â, Ntatsi, G., Petrotos, K., Barros, L., and Ferreira, I. (2018). Nutritional value, chemical characterization and bulb morphology of greek garlic landraces. *Molecules* 23:319. doi: 10.3390/molecules23020319

Petropoulos, S. A., Fernandes, Â, Barros, L., Ferreira, I. C., and Ntatsi, G. (2015). Morphological, nutritional and chemical description of "Vatikiotiko", an onion local landrace from Greece. *Food Chem.* 182, 156–163. doi: 10.1016/J.FOODCHEM.2015.03.002

Pike, L. (1986). "Onion breeding," in *Breeding Vegetable Crops*, ed. M. Bassett (Hartford, CT: AVI Publishing, Inc), 357–394.

Prakash, D., Singh, B. N., and Upadhyay, G. (2007). Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). *Food Chem.* 102, 1389–1393. doi: 10.1016/j.foodchem.2006.06.063

Prior, R. L., Wu, X., and Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 53, 4290–4302. doi: 10.1021/jf0502698

Puizina, J. (2013). Shallots in Croatia – genetics, morphology and nomenclature. *Acta Bot. Croat.* 72, 387–398. doi: 10.2478/botcro-2013-0016

Puizina, J., Javornik, B., Bohanec, B., Schweizer, D., Maluszynska, J., and Papeš, D. (1999). Cytogenetical evidences for hybrid structure and origin of diploid and triploid shallots (*Allium cepa* var.viviparum, Liliaceae) from Dalmatia (Croatia). *Genome* 42, 1208–1216. doi: 10.1139/g99-023

Puizina, J., and Papeš, D. (1996). Cytogenetical evidences for hybrid structure and origin of diploid and triploid shallots (*Allium cepa* var.viviparum, Liliaceae) from Dalmatia (Croatia). *Plant Syst. Evol.* 199, 203–215. doi: 10.1007/BF00984905

Rabinowitch, H. D., and Kamenetsky, R. (2002). "Shallot (*Allium cepa*, aggregatum group)," in *Allium Crop Science: Recent Advances*, eds H. D. Rabinowitch and L. Currah (Wallingford: CABI), 409–430. doi: 10.1079/9780851995106.0409

Rice-Evans, C., Miller, N., and Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends Plant Sci.* 2, 152–159. doi: 10.1016/S1360-1385(97)01018-2

Riggi, E., Avola, G., Siracusa, L., and Ruberto, G. (2013). Flavonol content and biometrical traits as a tool for the characterization of "cipolla di giarratana": a traditional sicilian onion landrace. *Food Chem.* 140, 810–816. doi: 10.1016/j.foodchem.2012.10.134

Schlesier, K., Harwat, M., Böhm, V., and Bitsch, R. (2002). Assessment of antioxidant activity by using different in vitro methods. *Free Radic. Res.* 36, 177–187. doi: 10.1080/10715760290006411

Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144–158.

Siracusa, L., Avola, G., Patanč, C., Riggi, E., and Ruberto, G. (2013). Re-evaluation of traditional mediterranean foods. The local landraces of "Cipolla di Giarratana" (*Allium cepa* L.) and long-storage tomato (*Lycopersicon esculentum* L.): quality traits and polyphenol content. *J. Sci. Food Agric.* 93, 3512–3519. doi: 10.1002/jsfa.6199

Slimestad, R., Fossen, T., and Vågen, I. M. (2007). Onions: a source of unique dietary flavonoids. *J. Agric. Food Chem.* 55, 10067–10080. doi: 10.1021/jf0712503

Soininen, T. H., Jukarainen, N., Auriola, S. O. K., Julkunen-Tiitto, R., Karjalainen, R., and Vepsäläinen, J. J. (2014). Quantitative metabolite profiling of edible onion species by NMR and HPLC-MS. *Food Chem.* 165, 499–505. doi: 10.1016/j.foodchem.2014.05.132

Stearn, W. T. (1992). How many species of allium are known? *Curtis's Bot. Mag.* 9, 180–182. doi: 10.1111/j.1467-8748.1992.tb00096.x

Takhtajan, A. L. (2009). *Flowering Plants*. 2nd edn. Dordrecht: Springer, doi: 10.1007/978-1-4020-9609-9

Treutter, D. (2010). Managing phenol contents in crop plants by phytochemical farming and breeding—visions and constraints. *Int. J. Mol. Sci.* 11, 807–857. doi: 10.3390/ijms11030807

USDA (2018). *USDA National Nutrient Database for Standard Reference*. Available at: https://ndb.nal.usda.gov/ndb/.

Vadalà, R., Mottese, A., Bua, G., Salvo, A., Mallamace, D., Corsaro, C., et al. (2016). Statistical analysis of mineral concentration for the geographic identification of garlic samples from Sicily (Italy), Tunisia and Spain. *Foods* 5:20. doi: 10.3390/foods5010020

Wright, J. S., Johnson, E. R., and DiLabio, G. A. (2001). Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J. Am. Chem. Soc.* 123, 1173–1183. doi: 10.1021/ja002455u

Yang, J., Meyers, K. J., van der Heide, J., and Liu, R. H. (2004). Varietal differences in phenolic content and antioxidant and antiproliferative activities of onions. *J. Agric. Food Chem.* 52, 6787–6793. doi: 10.1021/jf0307144

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 October 2018
doi: 10.3389/fpls.2018.01465

Improving the Commercial Value of the 'Calçot' (*Allium cepa* L.) Landrace: Influence of Genetic and Environmental Factors in Chemical Composition and Sensory Attributes

Silvia Sans[1,2]*, *Joan Casals*[1,2] *and Joan Simó*[1,2]

[1] *Miquel Agustí Foundation, Castelldefels, Spain,* [2] *Department of Agri-Food Engineering and Biotechnology, Universitat Politecnica de Catalunya, Castelldefels, Spain*

OPEN ACCESS

Edited by:
Lillian Barros,
Polytechnic Institute of Bragança, Portugal

Reviewed by:
Nikos Tzortzakis,
Cyprus University of Technology, Cyprus
Anestis Karkanis,
University of Thessaly, Greece

***Correspondence:**
Silvia Sans
silvia.sans.molins@upc.edu

Specialty section:
This article was submitted to Agroecology and Ecosystem Services, a section of the journal Frontiers in Plant Science

Received: *05 June 2018*
Accepted: *14 September 2018*
Published: *04 October 2018*

Citation:
Sans S, Casals J and Simó J (2018) Improving the Commercial Value of the 'Calçot' (Allium cepa L.) Landrace: Influence of Genetic and Environmental Factors in Chemical Composition and Sensory Attributes. Front. Plant Sci. 9:1465. doi: 10.3389/fpls.2018.01465

Landraces are considered valuable for their close ties to local cultures, adaptation to low inputs, and quality. 'Calçots' are the immature floral stems of second-year sprouts of onions from the 'Blanca Tardana de Lleida' landrace. 'Calçots' grown in their traditional area of cultivation have been awarded Protected Geographic Indication (PGI) 'Calçot de Valls' from the European Union. Despite annual sales of about €15 million, 'calçot' germplasm and cultivation methods are under-researched. This study aimed to estimate the influence of genetic and environmental factors in the chemical and sensory characteristics of 'calçots' to enable strategies to improve their commercial value to be devised. To this end, we tested the landrace and three new, more productive varieties derived from the landrace in experiments conducted over two seasons in six locations (within and outside the PGI zone), using two planting dates and two harvesting times. The results point to a major environmental influence in the quality of 'calçots.' The analysis of variance found all factors related with environmental influence were significant in most chemical traits considered (dry matter content, soluble solids content, pH, titratable acidity, and ash content), while the variety factor was significant only for titratable acidity. In sensory analyses, the variety factor and all the environmental factors had significant effects in all sensory traits recorded (sweetness, fiber perception, and off-flavors). In both chemical and sensory traits, most significant interactions involved the environmental factors. The negative correlation found between sweetness and fiber perception and off-flavors suggests that additional selection can bring 'calçots' closer to the sensory ideotype. Although clearly more productive, the new 'calçot' varieties maintain the chemical composition and sensory value of the landrace. Thus, fine-tuning the cultivation and/or breeding of the landrace for both yield and quality seem viable approaches to obtaining better commercial products.

Keywords: landrace, 'calçot', onion, chemical composition, sensory attributes, environmental influence

INTRODUCTION

Landraces are important resources in agriculture for their adaptation to particular environments and low inputs, their close ties to local cultures, and their tolerance and resistance to biotic and abiotic stresses (Newton et al., 2010; Casañas et al., 2017). Landraces renowned for their high sensory quality have maintained a commercial role in specialist production for niche markets (Villa et al., 2005). Extensive discussions have sought to define the concept of landrace, and many authors have associated landraces with a lack of formal genetic improvement (Zeven, 1998; Villa et al., 2005). Recently, Casañas et al. (2017) proposed to define landraces as cultivated varieties that have evolved and may continue to evolve through the use of conventional or modern breeding techniques in traditional or new agricultural environments within a defined ecogeographical area under the influence of the local human culture.

To recognize the added value of high quality local products and enhance rural development, the European Union promotes three types of food quality labels: Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Speciality Guaranteed (TSG). When applied to vegetables, PDO and PGI are closely tied to the geographical area of production and to the genotype-by-environment (GxE) interaction, which usually involves landraces. Thus, these designations simultaneously promote rural development and the *in situ* conservation of landraces (Smale et al., 2004). These food quality labels also help consumers identify products and crop varieties associated with cultural or biological heritage within a limited geographical area (Veteläinen et al., 2009). Products under food quality labels have singular organoleptic and/or nutritional traits derived from historically selected GxE interactions. To enhance these traits, some quality labels are incorporating descriptive sensory analysis through trained panels for quality control to ensure the sensory characteristics of their products (Pérez-Elortondo et al., 2018).

'Calçots' are the immature floral stems of second-year onion (*Allium cepa* L.) resprouts, mainly from the long-day 'Blanca Tardana de Lleida' (BTL) landrace, typically roasted on a hot open fire in Catalonia (Northeast Spain). The BTL landrace is characterized by late development, white skin and flesh, and the production of between 1 and more than 25 resprouts ('calçots') per onion. The European Union has designated the PGI 'Calçot de Valls' for 'calçots' from the BTL landrace of onions cultivated in the traditional area of cultivation (EC No 905/2002, 2002). There are no official economic data about 'calçots,' but it is estimated that the current market volume is about €15 million. Moreover, agro-tourism related with 'calçots' boosts the regional economy and has increased interest in demand for 'calçots' worldwide. The recent surge in commercialization has made farmers more interested in improving the quality and homogeneity of their product. To date, the regulating board of the PGI 'Calçot de Valls' has focused quality control on parameters related to external appearance (length and width of the edible part), but producers trading under the label aim to expand quality control to include quality-related parameters.

The agronomic performance of 'calçots' has been studied, and some tools have been developed to facilitate breeding for yield (Simó et al., 2013). As a result, two new more productive varieties have been obtained: Roquerola, which provides 320% more commercial-sized 'calçots' in early harvests, and Montferri, which provides 116% more 'calçots' in late harvests compared with the base population (Simó et al., 2012a). In parallel, a sensory ideotype has been elaborated; the ideal 'calçot' should have a high level of sweetness, low fiber perception, and no off-flavors (Simó et al., 2012b). In recent years, farmers of PGI 'Calçot de Valls' have relied heavily on the new varieties, but some historical populations are still cultivated. No breeding programs have been developed to improve the sensory quality of 'calçot' crop.

The chemical and nutritional composition, as well as the sensory profile of the plants, is determined by genetic and environmental factors and their interactions (Allard, 1999). In PDO or PGI products, the specific quality profile is conferred by the interaction between the genotype (i.e., landraces) and the environment (i.e., the historical area of production) (Romero del Castillo et al., 2008), and research programs should identify the genetic and environmental factors underlying these traits. For this reason, it is important to conduct studies that increase our understanding of the factors influencing quality.

As a first step toward expanding the attributes specified in the PGI 'Calçot de Valls' to include sensory traits, this study aimed to estimate the influence of genetic and environmental factors in some key chemical and sensory traits of 'calçots.'

MATERIALS AND METHODS

Experimental Design

Field experiments were conducted in two consecutive seasons (2014–2015 and 2015–2016) at six different locations with different pedo-climatic conditions in Catalonia (Northeast Spain) that represent standard 'calçot' production areas (**Figure 1**). Four experimental fields were located within the geographical area designated by the PGI, while the two others were outside the designated area.

Four different varieties of BTL were evaluated: the original population, which has not undergone any formal scientific breeding processes, the improved varieties Roquerola and Montferri, derived from the historical landrace by scientific breeding (Simó et al., 2012a), and a new experimental variety with higher yields, also derived from the historical landrace by scientific breeding. To avoid the effect of the first growing cycle conditions on 'calçots' cultivation, bulb onions used in the experiments were produced in the same field (Simó et al., 2013). In both experimental seasons, bulb onions were replanted at a density of 32,000 plants per hectare, using a planting pattern of 0.3 × 0.75 m, at two different times, in mid-August (early planting) and in late September (late planting). 'Calçots' were harvested at two different times, in December (early harvest) and in February (late harvest). The experimental design was three randomized blocks, with 50 plants per plot. Each experimental field was managed by farmers using their own customary traditional cultivation techniques. The fertilization, irrigation,

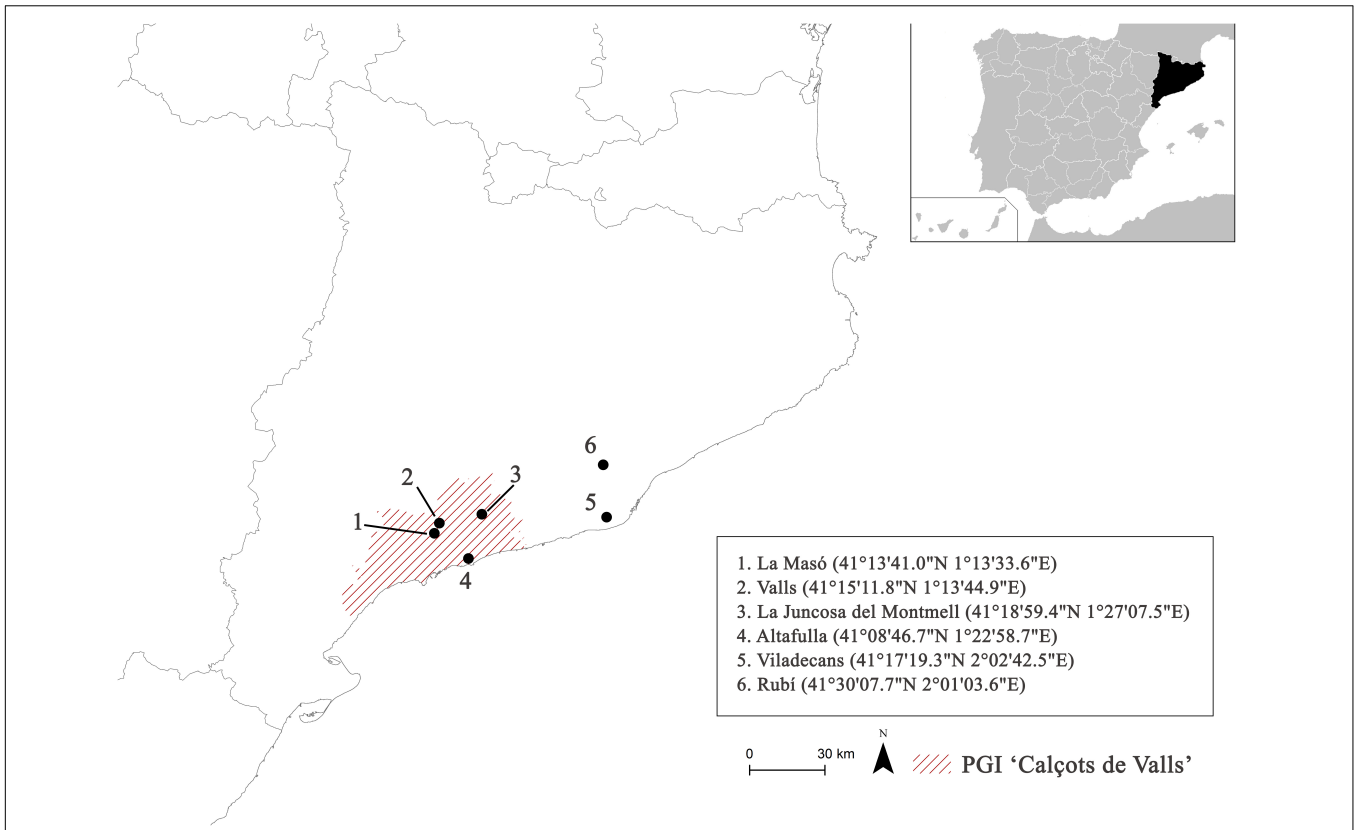

FIGURE 1 | Map of the experimental field locations.

TABLE 1 | Soil properties at the six locations in the two seasons.

Trait	Season	Altafulla	La Juncosa	La Masó	Rubí	Valls	Viladecans
Soil pH	14–15	8.54	8.47	8.38	8.43	8.27	8.57
	15–16	8.61	8.40	8.37	8.48	8.28	8.43
Electrical conductivity 25°C (dS m^{-1})	14–15	0.232	0.152	0.194	0.19	0.194	0.265
	15–16	0.212	0.174	0.223	0.18	0.22	0.275
Organic matter (%)	14–15	1.52	1.63	1.72	1.71	1.76	0.71
	15–16	1.12	1.30	1.34	1.28	1.3	1.10
Calcium carbonate equivalent (%)	14–15	36	38	39	23	52	24
	15–16	42	47	38	21	51	32
Nitrogen (mg N-NO3 kg^{-1})	14–15	40	18	32	36	78	22
	15–16	22	14	23	69	39	47
Phosphorus (mg kg^{-1})	14–15	20	21	27	18	3	9
	15–16	37	36	95	33	16	49
Potassium (mg kg^{-1})	14–15	230	590	374	334	292	547
	15–16	155	626	340	205	261	293
Calcium (mg kg^{-1})	14–15	6,529	6,979	6,456	6,555	7,066	7,150
	15–16	5,989	6,828	6,032	6,066	6,689	6,863
Magnesium (mg kg^{-1})	14–15	593	705	379	280	374	329
	15–16	499	531	344	300	358	337
Sodium (mg kg^{-1})	14–15	89	25	43	101	26	142
	15–16	157	17	35	41	44	104
Cation exchange capacity (cmol kg−1)	14–15	7.1	14.2	8.0	11.3	9.8	6.8
	15–16	6.3	15.7	7.5	11.8	10.4	9.9

TABLE 2 | Climatic data (°C) of the six locations in the two experimental seasons.

Location	Season	August		September		October		November		December		January		February	
		Mean min.	Mean max.	Mean min.	Mean max.	Mean min.	Mean max.	Mean min.	Mean max.	Mean min.	Mean max.	Mean min.	Mean max.	Mean min.	Mean max.
Altafulla	14–15	21.2	28.3	19.7	26.9	15.1	24.3	11.4	19.7	5.7	15.3	4.8	14.7	4.4	14.4
	15–16	20.9	29.3	17.2	25.3	13.7	22.2	9.5	19.3	8.2	16.7	7.5	16.2	6.8	17.0
La Juncosa	14–15	16.7	28.3	15.9	26.1	13.8	24.1	9.0	15.9	4.8	12.4	3.6	12.5	2.1	11.4
	15–16	17.0	29.6	13.5	24.2	10.9	20.4	8.5	18.1	7.1	15.6	5.3	13.4	5.2	14.1
La Masó	14–15	20.1	30.0	18.5	28.7	14.6	24.5	10.3	18.5	5.5	12.7	4.3	13.3	4.1	13.9
	15–16	20.4	31.1	16.6	25.6	12.5	21.6	9.1	17.7	7.1	15.6	6.3	15.5	6.4	16.1
Rubí	14–15	18.8	29.9	17.2	27.9	13.5	25.9	8.8	18.1	3.4	13.0	2.3	13.7	2.0	14.2
	15–16	18.6	31.4	15.1	26.2	11.7	22.6	7.0	19.4	5.0	16.4	5.1	15.8	4.8	17.2
Valls	14–15	17.6	28.1	16.4	26.7	12.4	24.3	8.3	17.7	3.5	13.0	2.2	12.9	2.0	12.7
	15–16	17.8	29.1	13.8	24.3	10.5	21.0	6.8	17.6	4.9	15.6	4.8	14.6	4.4	15.2
Viladecans	14–15	20.2	28.9	18.9	27.2	14.8	24.6	10.4	19.2	5.5	14.6	4.3	14.7	4.0	14.2
	15–16	20.2	30.6	16.8	26.1	13.2	22.6	8.9	19.6	7.2	17.1	6.4	16.4	5.7	17.3

Mean min.: mean of monthly minimum temperatures; Mean max.: mean of monthly maximum temperatures.

weed control and pest management were also managed using the farmer cultivation practices.

Soil Characteristics and Climate Data

Before planting, soil analyses were performed for each location. A hollow cylindrical corer with an internal diameter of 7 cm was used to collect seven 25-cm deep subsamples along a zigzag path from each experimental field. Subsamples were mixed to obtain homogeneous samples of about 1000 g for each site. The analyses were performed to evaluate the following soil properties: pH, electrical conductivity, percentage of organic matter, percentage of calcium carbonate equivalent, content of N, P, K, Ca, Mg, and Na, USDA textural class, and cation exchange capacity.

Climatic data (mean maximum and minimum monthly temperatures) were obtained from meteorological stations located near the experimental fields (**Table 2**). Rainfall was not considered because there was no unusual episode of rain and all the fields were cultivated under irrigation.

Sample Preparation

For each treatment (combination of variety, year, location, planting date, and harvest date) we collected three different samples. Each sample comprised a set of 80 commercial 'calçots' (PGI regulations define commercial 'calçots' as having a compact white edible base measuring 15–25 cm in length and 1.7–2.5 cm in diameter 5 cm from the root). 'Calçot' samples were prepared as described by Simó et al. (2012b). Leaves were cut 4 cm above the ligule, and roots were removed. Then, 'calçots' were rinsed with tap water to remove adhered soil and roasted at 270°C for 18 min in a convection oven (SALVA Kwik-co). After cooking, the two most external leaves were removed and the edible lower white part of each 'calçot' was cut. All 'calçots' in each sample were triturated with a mixer (Taurus BAPI 850). Pureed samples were frozen with liquid nitrogen and stored at −20°C until their chemical and sensory analyses.

Chemical Analysis

Soluble solids content was directly determined in the puree with a hand refractometer (Erma, Japan) and expressed as ^{o}Brix. To analyze titratable acidity, 10 g of puree was mixed with 50 mL of distilled water, initial pH was recorded, and then the mixture was titrated with 0.1 M sodium hydroxide (NaOH) to pH 8.1; titratable acidity was expressed as g/100 g of malic acid. To determine dry matter, 30 g of puree was dried to a constant weight for 72 h at 60°C; dry matter was expressed as g/100 g of fresh matter. To determine ash content, we used AOAC method 923.03 (AOAC, 2005): dried samples were ground to an average particle size < 0.4 mm to obtain flour; then, 1 g of flour was burned in a muffle at 450°C for 4 h, cooled to room temperature in a desiccator, and finally weighed. Ash was expressed as g/100 g of dry matter. All chemical analyses were carried out in triplicate.

Sensory Analysis

Descriptive sensory analysis requires trained panels, and these panels can work with a limited number of samples. This limitation precluded panel analysis of the nearly 200 samples

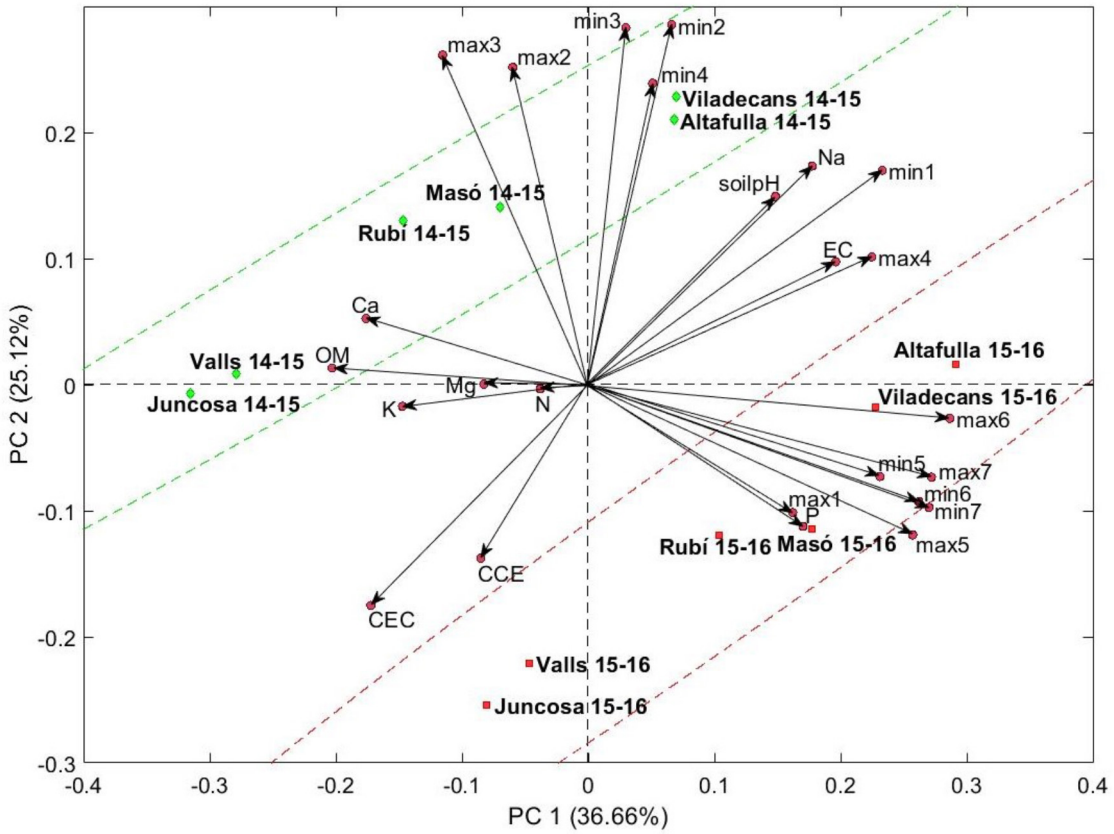

FIGURE 2 | Biplot of the location × season in the plane determined by the first two axes of the PCA considering the soil characteristics and climatic conditions. The angle of the vector with the axes indicates the correlation between the principal component and the original variable, and its length is proportional to the variability in the original variable explained by each principal component. The percentages between parentheses refer to the variation explained by each principal component. soilpH, pH of the soil; EC, electrical conductivity; OM, organic matter; CCE, calcium carbonate equivalent; N, nitrogen; P, phosphorus; K, potassium; Ca, calcium; Mg, magnesium; Na, sodium; CEC, cation exchange capacity; min, mean of the minimum temperatures for each month; max, mean of the maximum temperatures for each month; 1, 2, 3, 4, 5, 6, and 7 indicate August, October, November, December, January, and February, respectively.

generated along the experiment; therefore, sensory analysis consisted only of a preliminary survey using selected samples from the second year. Thus, the panel tested a subset of 32 samples representing the early and late harvests of the 4 varieties in 4 locations (La Masó, La Juncosa del Montmell, Valls, and Viladecans).

Sensory analysis was carried out as described by Simó et al. (2012b). Each of the 8 trained panelists evaluated the samples of puree in duplicate in a total of 13 sessions. Sensory attributes (sweetness, fiber perception, and off-flavors) were measured on semi-structured visual scales labeled from 0 to 10. All tests were carried out in a room designed for sensory tests that fulfilled the standards set out by the International Organization for Standardization (ISO 8589, 2007).

Statistical Analysis

Data were analyzed with R statistic software (R Core Team, 2017). PLS_Toolbox v.8.21 software (Eigenvector Research Inc., Wenatchee, WA, United States) was used for principal components analysis (PCA).

Each chemical and sensory trait was studied by ANOVA to detect statistical significance, according to the following linear models:

For chemical attributes,

$$X_{ijklm} = \mu + v_i + l_j + s_k + p_l + h_m + v_i l_j + v_i s_k + v_i p_l + v_i h_m + l_j s_k + l_j p_l + l_j h_m + s_k p_l + s_k h_m + p_1 h_m + \varepsilon_{ijklm}, \quad (1)$$

and for sensory attributes,

$$X_{ijkl} = \mu + v_i + l_j + h_k + t_l + v_i l_j + v_i h_k + v_i t_l + l_j h_k + l_i t_l + h_k t_l + \varepsilon_{ijkl}, \quad (2)$$

where v, l, s, p, h, and t are the factors variety, location, season, planting date, harvesting time, and trained panelist, respectively. All factors were considered fixed. Means of significant factors were compared by calculating the least significant difference (LSD) ($P < 0.05$). We used Pearson's correlation coefficient and regression to study the relations among the traits.

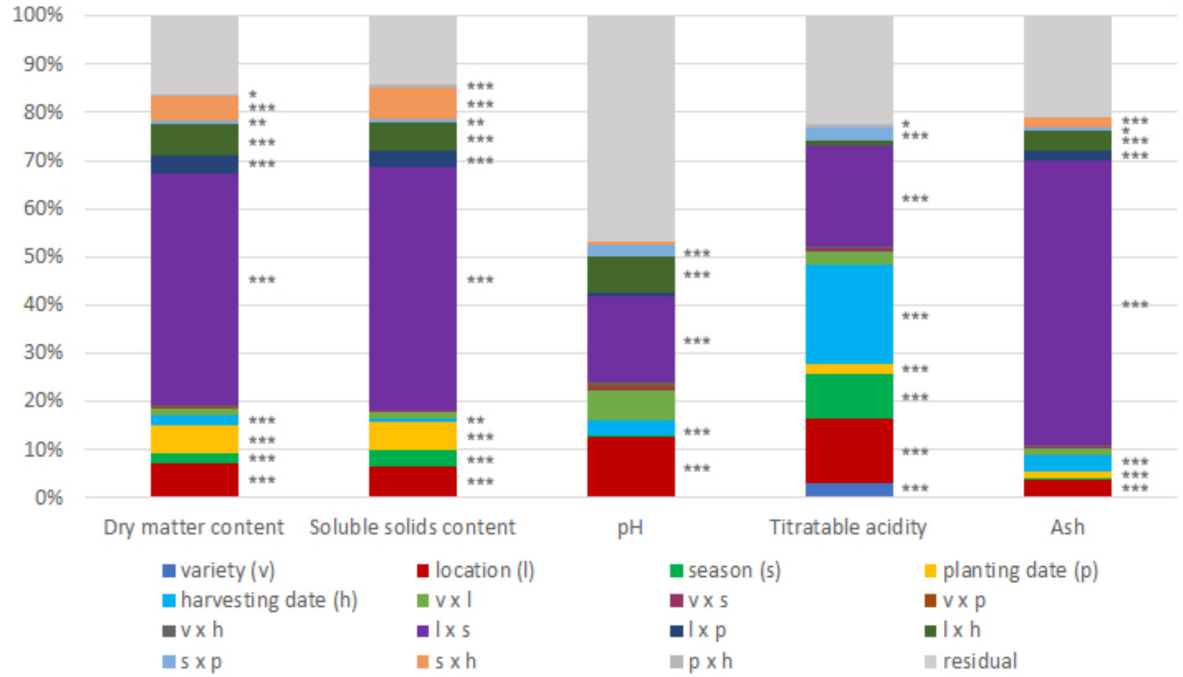

FIGURE 3 | Percentage of variance due to the factors considered in the ANOVA (n = 576) and their interactions in the chemical parameters studied. Significance codes of the factors considered in the ANOVA: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

RESULTS

Environmental Description

Soil texture was classified as loamy in all the experimental fields. The results of the remaining parameters studied (**Table 1**) showed that in general all the locations were calcareous, presented between basics and slightly alkaline soils and low concentration of organic matter, especially Viladecans during the first season (0.71%). In no case electrical conductivity was a limiting factor for 'calçot' cultivation.

Temperatures at all locations were characteristic of the mild Mediterranean climate, with marked differences over the months (**Table 2**). In general, the 2014-2015 season was warmer during the autumn, but in winter the highest temperatures were recorded in the 2015–2016 season.

Principal components analysis was performed on environmental characteristics (soil and climate data). The first three principal components, accounting for 74.9% of the total variance, revealed strong differences between seasons. The first component (PC1, 36.7%) was primarily correlated positively by mean minimum temperatures in December, January, and February; mean maximum temperatures in November, December, January, and February; sodium content; and electrical conductivity. Three factors correlated negatively with the first component: calcium content, cation exchange capacity, and organic matter. The traits that correlated positively most strongly with the second component (PC2, 25.1%) were mean minimum temperatures in August, September, October, and November; mean maximum temperatures in September and October; and soil pH. Two factors correlated negatively with the second component: cation exchange capacity and calcium carbonate equivalent. PCA revealed similarities among some locations, grouping them in pairs: Altafulla and Viladecans, La Masó and Rubí, and Valls and La Juncosa. However, the effect of the season seemed stronger since there was a clear displacement of all the locations between the two seasons studied, due to higher content of P and higher temperatures during the winter months (**Figure 2**).

Chemical Attributes

The analysis of variance showed a major environmental influence in chemical traits of 'calçots.' All factors related with environmental influence (location, planting date, harvesting time, and season) were significant ($P < 0.05$) for all the chemical traits considered, except the factors season and planting date for pH and the factor season for ash content. By contrast, the factor variety was significant only for the attribute titratable acidity. The only significant interactions were between factors related with environmental influence, being the interaction location × season the most important (**Figure 3**).

The greatest differences were found between locations (**Table 3**). Differences between locations ranged from 1.9% for pH to 34.6% for titratable acidity. Valls, La Juncosa, and Altafulla had the highest values for dry matter and soluble solids content, while Rubí had the lowest mean values for titratable acidity and ash content. As mentioned above, the only chemical trait that was significantly different between varieties was titratable acidity, which was highest in the "traditional" landrace and

lowest in the new "experimental" variety (**Table 3**). The amount of dry matter and soluble solids content were higher in the first season (2014–2015), and titratable acidity was higher in the second season (2015–2016). On average, 'calçots' planted early (in August) presented higher values of dry matter and soluble solids contents and lower values of titratable acidity and ash content. 'Calçots' from the early harvest presented the highest values for all the chemical parameters analyzed except pH.

Relationships Between Environmental Variables and Chemical Composition of 'Calçots'

Direct correlations were calculated between means of location × season of chemical parameters and environmental characteristics. Correlations were not robust, due to the complexity of the environmental factors. The only significant correlations ($P < 0.05$) were between soil pH and soil sodium content with the chemical trait pH of 'calçots' ($R = 0.8$ and $R = 0.61$, respectively) and between calcium carbonate equivalent and titratable acidity ($R = 0.59$).

Principal components analysis was applied using the means of chemical parameters in conjunction with environmental data (soil characteristics and climate data) of the 12 location × season combinations (**Figure 3**). The first three components explained 67.2% of the total variance, less than the PCA performed only with the environmental data (**Figure 2**). The two first components (PC1, 31.7%; PC2, 22.2%) were principally influenced by environmental characters (**Figure 4**) and were not notably different from the PCA that did not include the chemical parameters (**Figure 2**). The third component (PC3, 13.3 %) was strongly influenced by the chemical parameters dry matter and soluble solids content, with positive correlations, and ash content, with a negative correlation.

Sensory Attributes

All the main factors (variety, location, and harvesting time) were highly significant ($P < 0.01$) for all sensory traits, and the interactions between those factors were significant too. The factor panelist was also significant for the three sensory attributes considered, but none of the interactions that included the panelist factor were significant. In contrast to chemical parameters, we found significant differences between varieties; however, the

TABLE 3 | Means and standard deviations of chemical attributes between varieties, locations, seasons, planting dates, and harvesting times.

Factor	Dry matter content (g/100 g f.m.)	Soluble solids content (ºBrix)	pH	Titratable acidity (g malic acid /100 g f.m.)	Ash (g/100 g d.m.)
Variety					
Roquerola	15.946 ± 0.249a	12.7 ± 0.2a	6.10 ± 0.01a	0.127 ± 0.004b	5.129 ± 0.088a
Montferri	15.898 ± 0.239a	12.8 ± 0.2a	6.09 ± 0.02a	0.124 ± 0.003bc	5.155 ± 0.109a
Traditional	16.033 ± 0.224a	12.8 ± 0.2a	6.11 ± 0.01a	0.133 ± 0.004a	5.147 ± 0.100a
Experimental	15.803 ± 0.238a	12.6 ± 0.2a	6.09 ± 0.02a	0.121 ± 0.003c	5.152 ± 0.089a
% variation	–	–	–	9.9%	–
Location					
La Masó	15.575 ± 0.248c	12.4 ± 0.2c	6.09 ± 0.02bc	0.135 ± 0.005b	5.261 ± 0.072ab
Valls	16.609 ± 0.352a	13.4 ± 0.3a	6.04 ± 0.01d	0.144 ± 0.005a	4.995 ± 0.092cd
La Juncosa	16.066 ± 0.166b	12.8 ± 0.1b	6.07 ± 0.01cd	0.120 ± 0.002c	5.327 ± 0.135a
Altafulla	16.320 ± 0.159ab	13.1 ± 0.1ab	6.15 ± 0.02a	0.125 ± 0.004c	5.157 ± 0.053abc
Viladecans	15.383 ± 0.330c	12.2 ± 0.3c	6.11 ± 0.01b	0.123 ± 0.004c	5.148 ± 0.138bc
Rubí	15.324 ± 0.355c	12.3 ± 0.3c	6.10 ± 0.02bc	0.107 ± 0.004d	4.835 ± 0.105d
% variation	8.4%	9.5%	1.9%	34.6%	10.2%
Season					
14–15	16.226 ± 0.159a	13.1 ± 0.1a	6.10 ± 0.01a	0.118 ± 0.002b	5.088 ± 0.075a
15–16	15.622 ± 0.171b	12.4 ± 0.2b	6.09 ± 0.01a	0.134 ± 0.002a	5.202 ± 0.060a
% variation	3.8%	5.6%	–	13.6%	–
Planting date					
August	16.153 ± 0.143a	13.0 ± 0.1a	6.09 ± 0.01a	0.125 ± 0.002b	5.089 ± 0.053b
September	15.291 ± 0.185b	12.1 ± 0.2b	6.11 ± 0.01a	0.131 ± 0.004a	5.301 ± 0.103a
% variation	5.6%	7.1%	–	4.8%	4.2%
Harvesting time					
Early	16.419 ± 0.220a	13.0 ± 0.2a	6.07 ± 0.01b	0.143 ± 0.002a	5.290 ± 0.075a
Late	15.634 ± 0.131b	12.6 ± 0.1b	6.11 ± 0.01a	0.117 ± 0.002b	5.062 ± 0.061b
% variation	5.0%	3.8%	0.6%	22.2%	4.5%

Within columns and for each factor, means followed by the same letter were not significant different at P ≤ 0.05 (least significant difference test). f.m., fresh matter; d.m., dry matter.

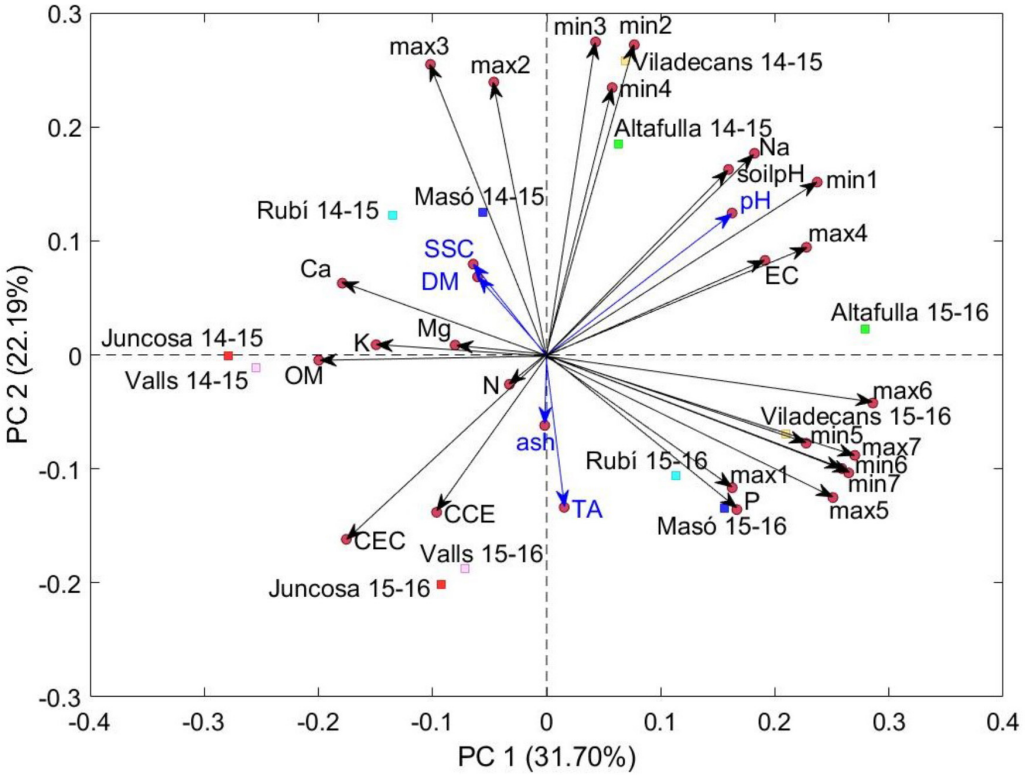

FIGURE 4 | Biplot of location × season in the plane determined by the first two axes of the principal component analysis considering the environmental data and chemical traits. The angle of the vector with the axes indicates the correlation between the principal component and the original variable, and its length is proportional to the variability in the original variable explained by each principal component. The percentages between parentheses refer to the variation explained by each principal component. DM, dry matter content; SSC, soluble solids content; TA, titratable acidity; soilpH, pH of the soil; EC, electrical conductivity; OM, organic matter; CCE, calcium carbonate equivalent; N, nitrogen; P, phosphorus; K, potassium; Ca, calcium; Mg, magnesium; Na, sodium; CEC, cation exchange capacity; min, mean of the minimum temperatures for each month; max, mean of the maximum temperatures for each month; 1, 2, 3, 4, 5, 6, and 7 indicate August, October, November, December, January, and February, respectively.

factor variety explained low percentages of the variation and was not the most influential factor in any sensory trait (**Figure 5**).

On average, the differences between varieties for sweetness were very low. Montferri had higher values for fiber perception and was among the varieties with highest values for off-flavors. Among locations, La Juncosa had the highest values for sweetness and lowest for off-flavors; Valls was the location with the lowest values for fiber perception. On average, 'calçots' from the late harvest had a sensory profile more in line with the ideotype, being sweeter and less fibrous, with less off-flavors (**Table 4**).

Correlations Among Chemical and Sensory Attributes

Overall, there were strong correlations between the chemical parameters dry matter, soluble solids, and ash content (**Table 5**). In contrast, pH and titratable acidity did not correlate with any of the other chemical parameters evaluated. Among the sensory attributes, we found negative correlations between sweetness and the other two (fiber, off-flavor). Analyzing the relationships between chemical and sensory parameters we included the ratio of soluble solids to titratable acidity (SSC/TA), since this ratio has been used to evaluate sweetness in some fresh produce (Magwaza and Opara, 2015). We found that sweetness was positively correlated with soluble solids, dry matter content and SSC/TA and negatively with ash content and titratable acidity. Fiber perception was positively correlated with ash content and negatively correlated with dry matter, soluble solids content and SSC/TA. Finally, the parameter off-flavors correlated positively with titratable acidity and ash content and negatively with the ratio SSC/TA.

DISCUSSION

The locations used for the experiment represented a wide range of variability on 'calçot' crop cultivation. In general, temperatures were the most variable parameters, especially between seasons. The differences in soil characteristics observed between locations can be attributed to the natural variation in soils throughout the territory and the differences in management practices among farmers (fertilization and soil tillage); these findings are representative of current 'calçot' production in Catalonia. Likewise, the

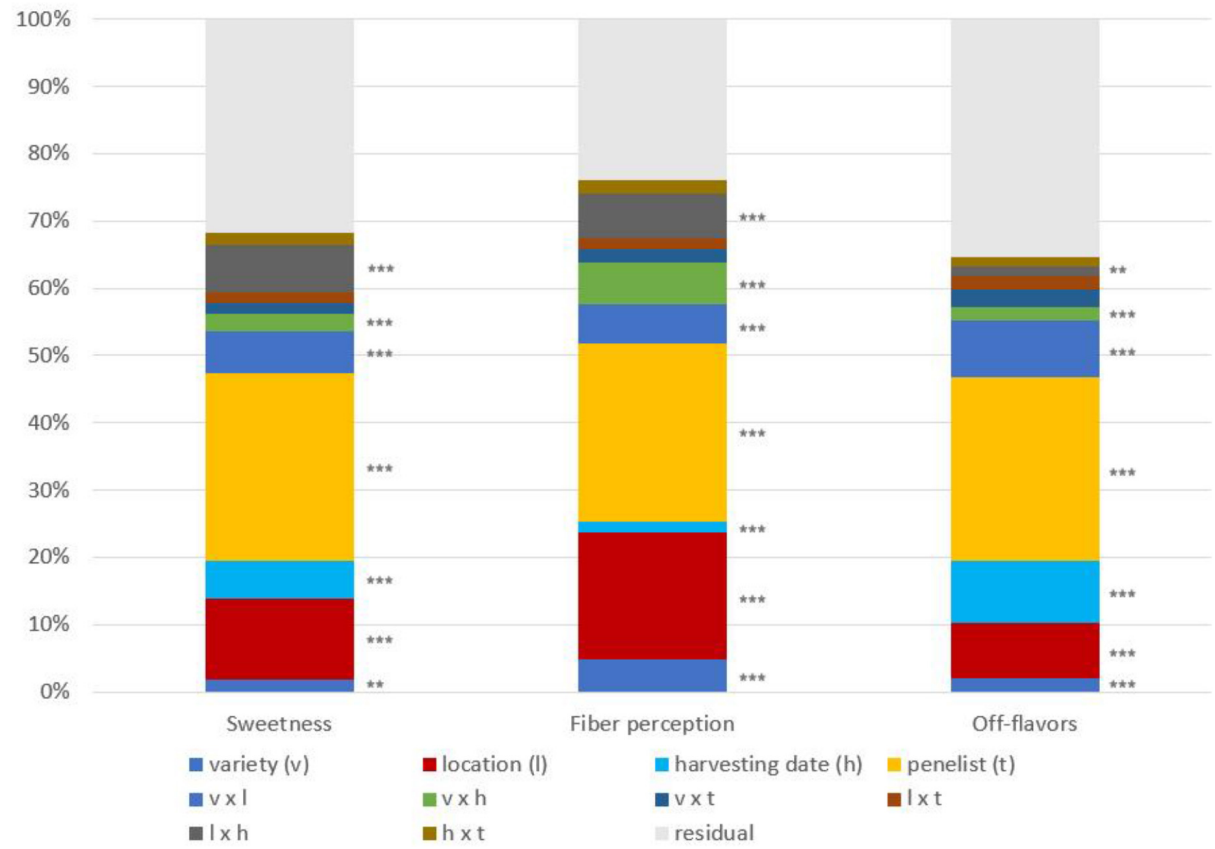

FIGURE 5 | Percentage of variance due to the factors considered in the ANOVA (n = 96) and their interactions in the sensory attributes studied. Significance codes of the factors considered in the ANOVA: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

varieties used in the experiment also are representative of current genotypic variability in farmers' 'calçot' fields.

Scant research related with quality in 'calçots' has been published. Zudaire et al. (2017) determined the pH, soluble solids content, and titratable acidity in the juice of raw 'calçot'; however, the methodological differences between their study and the current study make it difficult to compare results. Nevertheless, the orders of magnitude of the chemical parameters measured in our study were similar to those reported in roasted onions (Spanish food composition database, 2018) and raw onions (Barzegar et al., 2008; Petropoulos et al., 2015). Regarding the sensory analysis, the fact that the factor panelist was significant for all the traits considered indicates that panelists were applying the scales differently in their evaluations. The significance of the factor panelist is quite common in descriptive sensory analysis and it is related to slight differences in the reference values that judges learn (Romano et al., 2008). However, none of the interactions that included the panelist factor were significant, which means that, despite using different parts of the scale, the panel worked properly.

Our results show the important role of environmental factors in the chemical composition and sensory quality of 'calçots.' For all the chemical and sensory attributes studied, the effect of the environment was more important than the effect of the variety. Taking into account that our study is a first approximation, since no previous studies related with the influence of environmental variables or management practices have been done in 'calçot' cultivation, the results obtained will be helpful as a working basis for future research. The influence of environmental factors such as temperature, photoperiod, fertilization and/or other farming practices have been proved in the quality of crops (Hornick, 1992) such as onions (Sekara et al., 2017), tomatoes (*Solanum lycopersicum* L.) (Carli et al., 2011), globe artichoke (*Cynara scolymus* L.) (Lombardo et al., 2018) or leguminous vegetables (Ntatsi et al., 2018).

In our study, the role of genetic factors was less important. The chemical composition of 'calçots' from the four varieties studied was very similar, only differing in titratable acidity. Conversely, the factor variety was significant in sensory attributes. Sensory perception is highly complex, depending not only on chemical composition, but also on how volatile and non-volatile compounds interact, as has been studied in tomato (Baldwin et al., 2008). However, although the trained panel found statistically significant differences between the varieties for all the traits studied, these differences were limited in magnitude

and would probably be undetectable for untrained consumers. These findings of low variability were to be expected as the four varieties included in this experiment are of the same varietal type and the improved varieties were derived from breeding the historical landrace. The principal difference between the varieties of 'calçot' used is the number of resprouts per plant. Breeding programs developed for 'calçots' have used the variability within the landrace. Roquerola, Montferri, and the new experimental variety (150,489; 164,668 and 175,656 'calçots'/ha respectively) were clearly more productive than the traditional population in this experiment (125,403 'calçots'/ha). Importantly, our results indicate that selection for increased production did not have a negative impact on quality, perhaps because the sensory profile was taken into account in these breeding programs to improve production (Simó et al., 2012a), though chemical composition was not controlled. Likewise, the present results are important because they show that the breeding program did not have an important impact on the chemical parameters studied; thus, it seems that a synchronic improvement of yield and quality-related traits may be possible for 'calçots,' in contrast with the dilution effect described for many other species (Morris and Sands, 2006).

The factor location was the main source of variation in the chemical and sensory parameters studied. The influence of growing site on quality parameters has been proved also in onions (Mallor et al., 2011; Lee et al., 2016) as well as other crops such as raspberries (*Rubus idaeus*) (Castilho Maro et al., 2014) or beans (*Phaseolus vulgaris* L.) (Florez et al., 2009). Our study included locations outside the area designated in the PGI; however, we found no clear pattern differentiating between the chemical composition of 'calçots' grown inside the PGI area and those grown outside this area. Nevertheless, with respect to the sensory profile, the values for the sensory attributes of 'calçots' grown in Viladecans (outside the PGI area) were the farthest from the ideotype. This approach should be further investigated, as it can improve the robustness of the quality label, as has been done in other products, such as beans (Florez et al., 2009), olive oil (Cosio et al., 2006), or wine (Díaz et al., 2003).

The time of year when 'calçots' were planted and harvested also had an important influence on the quality of 'calçots.' It is important to point out that differences in harvesting time did not only involve different environmental conditions. 'Calçots' of the late harvest usually had a slower development, so the differences found between harvests may also be due to some genetic differences. The present study has been useful in showing that these two factors had an influence on 'calçots' quality; however, due to the complexity of the experiment and the interactions between the environmental factors studied, more focused experiments must be done before solid recommendations can be given to farmers.

The experimental design of this study included different locations with a combination of soil properties and temperature effects, and different planting and harvesting times, which in the end, also represented different environmental options at a certain moment in the plant life cycle. The complexity of the environmental factors and their interactions provide us with an overview of the influence of the environment on 'calçot' crop, which had been never studied before. However, such a broad study makes it difficult to disentangle specific findings.

TABLE 4 | Means and standard deviations of sensory attributes between varieties, locations, and harvesting times.

Factor	Sweetness	Fiber	Off-flavors
Variety			
Roquerola	6.8 ± 0.1a	1.8 ± 0.1c	2.1 ± 0.2a
Montferri	6.3 ± 0.2b	2.9 ± 0.2a	2.2 ± 0.2a
Traditional	6.5a ± 0.2b	2.1 ± 0.2bc	2.1 ± 0.2a
Experimental	6.6 ± 0.2a	2.3 ± 0.2b	1.6 ± 0.2b
% variation	8.4%	57.9%	43.2%
Location			
La Masó	6.6 ± 0.2b	1.9 ± 0.1c	2.4 ± 0.2a
Valls	6.8 ± 0.2b	1.6 ± 0.1d	1.9 ± 0.2b
La Juncosa	7.2 ± 0.1a	2.2 ± 0.2b	1.2 ± 0.1c
Viladecans	5.8 ± 0.1c	3.6 ± 0.2a	2.5 ± 0.2a
% variation	23.0%	131.0%	111.6%
Harvesting time			
Early	6.2 ± 0.1b	2.5 ± 0.1a	2.5 ± 0.2a
Late	6.9 ± 0.1a	2.1 ± 0.1b	1.4 ± 0.1b
% variation	10.8%	23.0%	76.4%

Season 2015–2016. Within columns and for each factor, means followed by the same letter were not significant different at $P \leq 0.05$ (least significant difference test).

TABLE 5 | Correlations between chemical and sensory traits.

	Fiber	Off-flavors	Dry matter	Soluble solids content	pH	Titratable acidity	Ash	SSC/TA
Sweetness	−0.52**	−0.74***	0.47**	0.52**	−0.12	−0.38*	−0.62***	0.59***
Fiber		0.28	−0.56***	−0.58***	−0.23	0.07	0.48**	−0.43*
Off-flavors			−0.21	−0.26	0.14	0.61***	0.44*	−0.60***
Dry matter				0.97***	−0.22	0.02	−0.87***	0.61***
Soluble solids content					−0.23	−0.01	−0.92***	0.65***
pH						0.12	0.35*	−0.21
Titratable acidity							0.29	−0.75***
Ash								−0.81***

*SSC/TA: ratio of soluble solids to titratable acidity. *, **, *** indicates significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively (Student's t-test).*

From the two PCAs (**Figures 2**, **3**) and the direct correlations between chemical parameters and environmental data, we could infer that the chemical parameters studied could be explained through linear combinations of some of the conditions controlled in the experiment, but this relation is not easily described by practical equations. However, temperatures seemed to play an important role on the chemical parameters studied. Moreover, soil properties that could be modified through fertilization had an impact on the 'calçots'; soil pH and sodium content influenced the pH of 'calçots' and, together with other cations, soil phosphorus content could influence the dry matter and soluble solids content of 'calçots.' The effect of both temperatures and fertilization in onion has been studied several times since these factors affect not only the plant development, but also the quality of the bulbs (Petropoulos et al., 2017; Sekara et al., 2017). The insights from this study allow us to speculate on future directions for research, but further studies will be required to grasp the complex factors underlying quality in 'calçots.'

Trying to understand which environmental characteristics had an important influence on sensory attributes is even more complex, since only a subset of samples was analyzed. It is unfeasible to analyze a large number of samples via sensory analysis with trained panelists, because they can only assess a limited number of samples per testing session (Plans et al., 2014). Therefore, other approaches are necessary. Establishing relationships between chemical composition and sensory traits opens the door to approaches that can deal with large numbers of samples. Among the correlations between chemical and sensory parameters found in the present study, the significant positive correlation between sweetness and soluble solids content, a chemical parameter that has been widely used to indicate sweetness of fresh and processed horticultural products, seems especially promising (Magwaza and Opara, 2015). The correlation between sweetness and the ratio SSC/TA has been slightly higher but considering the increase of work on the analysis, the use of soluble solids content seems to be a better approach. Correlations between chemical parameters and sensory traits can be useful for breeding programs or quality control, where it may be necessary to work with large numbers of samples that would be impossible for panels to evaluate.

There are two possible approaches to improving nutritional composition or quality characteristics of 'calçots': breeding or modifying cultivation conditions. Since the variability among 'calçots' varieties is low, intravarietal variation must be exploited (Simó et al., 2013) and, if necessary, other varieties might be used to introduce new variability. Moreover, the negative estimated genotypic correlations between sweetness and the other two sensory attributes (fiber perception and off-flavors, both of which are undesirable) suggest that additional selection can bring 'calçots' closer to the sensory ideotype. However, our results show that much work remains to increase knowledge and improve crop management through factors such as irrigation and fertilization management, incidence of pests and diseases, effects of weather, type of soil, or weed management.

CONCLUSION

The present study has generated new information regarding factors involved in the quality of 'calçots,' a crop barely investigated, enabling the influence of genetic and environmental factors in some key chemical and sensory traits of 'calçots' to be estimated.

Overall, the results point to a major environmental influence in the quality of 'calçots' cultivated from the most common varieties of BTL onion, including the original landrace. The low variability in the chemical composition and sensory traits among these varieties confirms that breeding programs to increase the production of 'calçots' plants have not significantly affected quality. Furthermore, this study has established correlations between sensory attributes and chemical parameters that can be useful when large numbers of samples need to be characterized in breeding or quality control. Finally, both breeding programs and crop management seem to be valid approaches to improving the commercial value of 'calçots.'

AUTHOR CONTRIBUTIONS

SS performed the chemical analysis, conducted the testing sessions, participated in the interpretation of data and drafting the manuscript, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JC revised the article critically, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JS made substantial contributions to the conception or design of the work, participated in the analysis and interpretation of data, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This research was carried out with the financial support of ACCIÓ (Generalitat of Catalonia, RD14-1-004), 'Cooperativa de Valls,' 'Cooperativa de Cambrils,' and PGI 'Calçot de Valls.' This work has been supported by the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement (FI-DGR 2015).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the farmers who provided their fields for the experiments. We also thank the members of the testing panel.

REFERENCES

Allard, R. W. (1999). *Principles of Plant Breeding*. New York, NY: John Wiley and Sons.

AOAC (2005). *Official Methods of Analysis*, 18th Edn. Gathersburg, MD: Association of Official Analytical Chemists.

Baldwin, E. A., Goodner, K., and Plotto, A. (2008). Interaction of volatiles, sugars, and acids on perception of tomato aroma and flavor descriptors. *J. Food Sci.* 73, S294–S307. doi: 10.1111/j.1750-3841.2008.00825.x

Barzegar, M., Jabbari, A., Rajabi, A., and Hassandokht, M. R. (2008). Chemical composition of different cultivars of onion (*Allium cepa* L.) produced in Iran. *Hortic. Environ. Biotechnol.* 49, 121–127.

Carli, P., Barone, A., Fogliano, V., Frusciante, L., and Ercolano, M. R. (2011). Dissection of genetic and environmental factors involved in tomato organoleptic quality. *BMC Plant Biol.* 11:58. doi: 10.1186/1471-2229-11-58

Casañas, F., Simo, J., Casals, J., and Prohens, J. (2017). toward an evolved concept of landrace. *Front. Plant Sci.* 8:145. doi: 10.3389/fpls.2017.00145

Castilho Maro, L. A., Pio, R., Santos Guedes, M. N., Patto de Abreu, C. M., and Abreu Moura, P. H. (2014). Environmental and genetic variation in the post-harvest quality of raspberries in subtropical areas in Brazil. *Acta Sci. Agron.* 36, 323–328. doi: 10.4025/actasciagron.v36i3.18050

Cosio, M. S., Ballabio, D., Benedetti, S., and Gigliotti, C. (2006). Geographical origin and authentication of extra virgin olive oils by an electronic nose in combination with artificial neural networks. *Anal. Chim. Acta* 567, 202–210. doi: 10.1016/j.aca.2006.03.035

Díaz, C., Conde, J. E., Estévez, D., Pérez Olivero, S. J., and Pérez Trujillo, J. P. (2003). Application of multivariate analysis and artificial neural networks for the differentiation of red wines from the Canary Islands according to the island of origin. *J. Agric. Food Chem.* 51, 4303–4307. doi: 10.1021/jf0343581

EC No 905/2002 (2002). *Commission Regulation (EC) No 905/2002 of 30 May 2002 Supplementing the Annex to Regulation (EC) No 2400/96 on the Entry of Certain Names in the 'Register of Protected Designations of Origin and Protected Geographical Indications' OJ L 142/2*. Brussels: European Commission.

Florez, A., Pujola, M., Valero, J., Centelles, E., Almirall, A., and Casañas, F. (2009). Genetic and environmental effects on chemical composition related to sensory traits in common beans (*Phaseolus vulgaris* L.). *Food Chem.* 113, 950–956. doi: 10.1016/j.foodchem.2008.08.036

Hornick, S. B. (1992). Factors affecting the nutritional quality of crops. *Am. J. Altern. Agric.* 7, 63–68. doi: 10.1017/S0889189300004471

ISO 8589 (2007). *Sensory Analysis – General Guidance for the Design of Test Rooms*. Geneva: International Organization for Standardization.

Lee, J., Ha, I., Kim, H., Choi, S., Lee, S., Kang, J., et al. (2016). Regional differences in onion bulb quality and nutrient content, and the correlation between bulb characteristics and storage loss. *Korean J. Hortic. Sci.* 34, 807–817. doi: 10.12972/kjhst.20160085

Lombardo, S., Pandino, G., and Mauromicale, G. (2018). The influence of pre-harvest factors on the quality of globe artichoke. *Sci. Hortic.* 233, 479–490. doi: 10.1016/j.scienta.2017.12.036

Magwaza, L. S., and Opara, U. L. (2015). Analytical methods for determination of sugars and sweetness of horticultural products-a review. *Sci. Hortic.* 184, 179–192. doi: 10.1016/j.scienta.2015.01.001

Mallor, C., Balcells, M., Mallor, F., and Sales, E. (2011). Genetic variation for bulb size, soluble solids content and pungency in the Spanish sweet onion variety Fuentes de Ebro. Response to selection for low pungency. *Plant Breed.* 130, 55–59. doi: 10.1111/j.1439-0523.2009.01737.x

Morris, C. E., and Sands, D. C. (2006). The breeder's dilemma - yield or nutrition? *Nat. Biotechnol.* 24, 1078–1080. doi: 10.1038/nbt0906-1078

Newton, A. C., Akar, T., Baresel, J. P., Bebeli, P. J., Bettencourt, E., Bladenopoulos, K. V., et al. (2010). Cereal landraces for sustainable agriculture. A review. *Agron. Sustain. Dev.* 30, 237–269. doi: 10.1051/agro/2009032

Ntatsi, G., Egea Gutierrez-Cortines, M., Karapanos, I., Barros, A., Weiss, J., Balliu, A., et al. (2018). The quality of leguminous vegetables as influenced by preharvest factors. *Sci. Hortic.* 232, 191–205. doi: 10.1016/j.scienta.2017.12.058

Pérez-Elortondo, F. J., Symoneaux, R., Etaio, I., Coulon-Leroy, C., Maître, I., and Zannoni, M. (2018). Current status and perspectives of the official sensory control methods in protected designation of origin food products and wines. *Food Control* 88, 159–168. doi: 10.1016/j.foodcont.2018.01.010

Petropoulos, S. A., Fernandes, A., Barros, L., Ferreira, I. C. F. R., and Ntatsi, G. (2015). Morphological, nutritional and chemical description of "Vatikiotiko", an onion local landrace from Greece. *Food Chem.* 182, 156–163. doi: 10.1016/j.foodchem.2015.03.002

Petropoulos, S. A., Ntatsi, G., and Ferreira, I. C. F. R. (2017). Long-term storage of onion and the factors that affect its quality: a critical review. *Food Rev. Int.* 33, 62–83. doi: 10.1080/87559129.2015.1137312

Plans, M., Simó, J., Casañas, F., Romero del Castillo, R., Rodriguez-Saona, L. E., and Sabaté, J. (2014). Estimating sensory properties of common beans (*Phaseolus vulgaris* L.) by near infrared spectroscopy. *Food Res. Int.* 56, 55–62. doi: 10.1016/j.foodres.2013.12.003

R Core Team (2017). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Romero del Castillo, R., Almirall, A., Valero, J., and Casanas, F. (2008). Protected designation of origin in beans (*Phaseolus vulgaris* L.): towards an objective approach based on sensory and agromorphological properties. *J. Sci. Food Agric.* 88, 1954–1962. doi: 10.1002/jsfa.3303

Romano, R., Brockhoff, P. B., Hersleth, M., Tomic, O., and Naes, T. (2008). Correcting for different use of the scale and the need for further analysis of individual differences in sensory analysis. *Food Qual. Prefer.* 19, 197–209. doi: 10.1016/j.foodqual.2007.06.008

Sekara, A., Pokluda, R., Del Vacchio, L., Somma, S., and Caruso, G. (2017). Interactions among genotype, environment and agronomic practices on production and quality of storage onion (*Allium cepa* L.) - A review. *Hortic. Sci.* 44, 21–42. doi: 10.17221/92/2015-HORTSCI

Simó, J., Romero del Castillo, R., Almirall, A., and Casañas, F. (2012a). "Roquerola" and "Montferri" first improved onion (*Allium cepa* L.) cultivars for "calçots" production. *Hortscience* 47, 801–802.

Simó, J., Romero del Castillo, R., and Casañas, F. (2012b). Tools for breeding 'calçots' (*Allium cepa* L.), an expanding crop. *Afr. J. Biotechnol.* 11, 11065–11073. doi: 10.5897/AJB12.904

Simó, J., Valero, J., Plans, M., Romero del Castillo, R., and Casañas, F. (2013). Breeding onions (*Allium cepa* L.) for consumption as "calçots" (second-year resprouts). *Sci. Hortic.* 152, 74–79. doi: 10.1016/j.scienta.2013.01.011

Smale, M., Bellon, M. R., Jarvis, D., and Sthapit, B. (2004). Economic concepts for designing policies to conserve crop genetic resources on farms. *Genet. Resour. Crop Evol.* 51, 121–135. doi: 10.1023/b:gres.0000020678.82581.76

Spanish food composition database (2018). *Spanish Food Composition Database*. Available at: http://www.bedca.net/bdpub/index_en.php [accessed Jan 11, 2018].

Veteläinen, M., Negri, V., and Maxted, N. (2009). *European Landraces on Farm Conservation, Management and Use*. Rome: Bioversity International.

Villa, T. C., Maxted, N., Scholten, M. A., and Ford-Lloyd, B. V. (2005). Defining and identifying crop landraces. *Plant Genet. Res.* 3, 373–384. doi: 10.1079/PGR200591

Zeven, A. C. (1998). Landraces: a review of definitions and classifications. *Euphytica* 104, 127–139. doi: 10.1023/A:1018683119237

Zudaire, L., Vinas, I., Abadias, M., Simo, J., Echeverria, G., Plaza, L., et al. (2017). Quality and bioaccessibility of total phenols and antioxidant activity of *calçots* (*Allium cepa* L.) stored under controlled atmosphere conditions. *Postharvest Biol. Technol.* 134, 122–123. doi: 10.1016/j.postharvbio.2017.03.013

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 February 2018
doi: 10.3389/fpls.2018.00170

Preliminary Evidences of Biofortification with Iodine of "Carota di Polignano", An Italian Carrot Landrace

Angelo Signore[1], *Massimiliano Renna*[1,2]*, *Massimiliano D'Imperio*[2], *Francesco Serio*[2] and *Pietro Santamaria*[1]

[1] Department of Agricultural and Environmental Science, University of Bari Aldo Moro, Bari, Italy, [2] Institute of Sciences of Food Production, National Research Council, Bari, Italy

The "*Carota di Polignano*" (Polignano Carrot – PC, *Daucus carota* L.) is a multi-colored landrace, cultivated in the Southern Italy, whose colors range from yellow to purple. Iodine is an essential micronutrient for humans, since it is a key component of thyroid hormones, which regulate the growth and development of the human body. The main source for iodine assumption is represented by diet, but its concentration in the vegetables is usually limited with respect to human needs. To this purpose, two experimental trials (in open field and in greenhouse with a soil-less system) were carried out to enrich PC with iodine. Three levels of iodine (control treatment, C – 0 mg·L^{-1}; low, L – 50 mg·L^{-1}; and high, H – 500 mg·L^{-1}), distributed with foliar spray fertilizations (in both open field and greenhouse) or with nutrient solution (in greenhouse, at the level of 50 mg·L^{-1}) in the form of KIO_3 were compared. In open field, the H treatment showed a biofortification that was double and triple respect to L and C treatments, respectively, without influencing color and biometric parameters, such as the fresh and dry weight of roots and DM percentage. In greenhouse, the biofortification done with foliar spray fertilization followed the same trend of open field, while the biofortification by means of nutrient solution was more effective but reached very high levels that had toxic effects on the plants and could be too high for human nutrition. However, the concentrations of iodine into biofortified carrots in open field can allow to satisfy the recommended daily allowance (RDA) by consuming 100 and 200 g of fresh product for the treatment H and L, respectively. Regarding the greenhouse biofortification, the RDA would be satisfied by consuming 200 g of fresh carrots (with the high level of foliar fertilization).

Keywords: ***Daucus carota*** **L., Polignano carrot, multi-colored roots, iodine RDA, soil-less**

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Georgia Ntatsi, Agricultural University of Athens, Greece
Juan A. Fernández, Universidad Politécnica de Cartagena, Spain

***Correspondence:**
Massimiliano Renna
massimiliano.renna@uniba.it

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Plant Science

Received: *20 December 2017*
Accepted: *30 January 2018*
Published: *12 February 2018*

Citation:
Signore A, Renna M, D'Imperio M, Serio F and Santamaria P (2018) Preliminary Evidences of Biofortification with Iodine of "Carota di Polignano", An Italian Carrot Landrace. Front. Plant Sci. 9:170. doi: 10.3389/fpls.2018.00170

INTRODUCTION

Italy is one of the most important vegetable-producing country in Europe, and the Puglia Region (Southern Italy), that forms about 22% of the Italian total vegetable-growing area, is the most important region in Italy for open field crops, with more than 100,000 hectares (Istituto Nazionale di Statistica, 2017[1]). Because of its particular conformation and position, the Puglia region holds

[1]Superfici Agricole Regione Puglia. http://agri.istat.it/—last access 19/12/2017.

a great heritage of agro-biodiversity, with particular reference to vegetables. Unfortunately, such agro-biodiversity has been partially lost, due to several factors (Elia and Santamaria, 2013; Signore, 2016). To counteract such loss, in a context of a project about agro-biodiversity, the Puglia Region Administration undertook several initiatives, with the aim to identify, protect and recover several landraces of vegetables at risk of genetic erosion (Renna et al., 2014). Among such landraces, there is a carrot called, in Italian, *Carota giallo-viola di Polignano* (yellow-purple carrot of Polignano—the municipality where it is located), a multicolored (ranging from yellow to purple tone) landrace of *Daucus carota* (L.), from here onward named PC, that has been previously studied and characterized for some parameters, such as the compositional and antioxidant profiles (Cefola et al., 2012). However, one of the main purposes of the Puglia Region Administration plan is to valorize such landrace(s), in order to push more and more farmers to cultivate them, and realize a full recovery of the agro-biodiversity. However, such valorization passes not only from the farmers, but also from consumers. Since the PC has higher prices on the market, up to 2–3-fold the "commercial" carrot, to stimulate its consumption is crucial to find an added value that could push the consumers to prefer the PC to the "commercial" one. An added value for a product may be represented by its nutritional aspects, either already present in its composition, or subsequently added.

A way to realize such added valued may be represented by the biofortification, a technique that consists in adding some (micro)nutrient(s), beneficial for human health, to food. From this point of view, iodine it is a perfect example, because is responsible, together with other minerals such as vitamin A and iron, of the "hidden hunger," defined by the WHO as "a lack of vitamins and minerals" (World Health Organization, 2004[2]). Iodine is an essential trace element for human, since it is a fundamental component of thyroid hormones that regulate the growth and development of the body, and its deficiency may strongly affect the functionality of thyroid, by the means of two iodine containing-hormones: triiodothyronine (T3) and thyroxine (T4). From this point of view, the iodine is a rate-limiting element for the synthesis of such hormones.

According with White and Broadley (2009), 30–38% of the world's population has insufficient iodine intake and live with risk for iodine deficiency, and associated iodine deficiency disorders (IDD). According to Global Iodine Network (2017)[3], such deficiency is present even in developed countries: Italy for example has an insufficient iodine intake. The iodine deficiency has been associated with several diseases, such as mental impairment and goiter in older children and adults (de Benoist et al., 2008) and complications with pregnancy, during which inadequate iodine intake may lead to irreversible brain damage to the fetus (World Health Organization, 2007).

For iodine, the Recommended Daily Allowance (RDA) value changes according to the Country, the Organization that suggests the RDA, the age and other factors such as, for example, being a pregnant or breastfeeding woman, with the values that normally range from 90 to 290 μg (Zimmermann, 2017). The problem of IDD is even more serious considering that the consumption of one of the most important sources for iodine, milk, has decreased since the 1950s, even if it has remained relatively steady in recent years, and milk alternatives have negligible iodine content, therefore are not appropriate substitutes in terms of iodine provision (Bath et al., 2017). To counteract the IDD, one of the most effective way is the iodization of kitchen salt but, even so, a third of the global population is still unprotected from iodine deficiency (World Health Organization, 2005; Gunnarsdottir and Dahl, 2012; European Food Safety Authority, 2014). Moreover, the iodine has low stability in salt and losses occur in the several steps of production, packaging, transportation and processing, hence the total amount of iodine lost from salt may reach 90%, with the cooking process that may contribute on average to 20% of such losses (Winger et al., 2008). Such losses can occur even in the food: for example the biofortified carrots may lose around 55% of their iodine content during the boiling process (Comandini et al., 2013). Besides, the excessive consumption of salt has some drawbacks, because it may cause problem of hypertension. According to Zimmermann and Andersson (2012), the current salt intake in children is unnecessarily high and is very likely to predispose children to develop hypertension later.

Thus, to overcome the salt-related problems, the biofortification should be done with food, specifically vegetables, that do not have the salt side effects, so they may be used as a vector for this mineral in the diet, since many of such products are consumed raw (Haldimann et al., 2005) and the iodine losses are usually negligible. Several vegetable crops can store iodine such as lettuce (Cerretani et al., 2014), spinach (Dai et al., 2006), tomato (Landini et al., 2011; Kiferle et al., 2013; Smolen et al., 2015) and carrot (Dai et al., 2004; Hong et al., 2008; Comandini et al., 2013; Smolen et al., 2016). Starting from the above premises, the scopes of our research were to: (i) enrich a local landrace of carrot with iodine, both in a traditional cultivation system (open field) and with an advanced system (soil-less system) and (ii) evaluate the effect of iodine concentration on the other quality parameters of the carrot root.

MATERIALS AND METHODS

Open Field—Crop System and Treatments

The trial was carried out between October 2013 and April 2014, in a field located in Polignano a Mare, Southern Italy (41.011111, 17.189694).

The soil of the experimental field, which distance from the sea was about 400 m, was basically sandy and had a concentration of total nitrogen of 1.3‰, while the concentration of organic matter was 2.33%.

The temperature ranged from 3.5° to 25°C and the percentage of relative humidity from 25 to 99% during the crop cycle (minimum/maximum respectively—data not shown).

The experimental treatments were arranged in a completely randomized design with three replications. Every plot was a square with a side of 3 m width having a border zone of 1 m.

[2]http://whqlibdoc.who.int/publications/2004/9241546123.pdf—last access 19/12/2017

[3]http://www.ign.org/cm_data/IGN_Global_Map_AllPop_30May2017.pdf—last access 19/12/2017

The sowing was done on October 15, by putting the seeds in continuous manner on the row (30–40 seeds·m^{-1}). The distance between rows was 0.35 m, resulting in a final density of 70–100 plants·m^{-2}, according with the common practice (for the details regarding sowing and other crop techniques, see the video in the "Supplementary Material" section).

The fertilization of the field was not necessary, since local farmers apply the agronomic principle of crop rotation, thus soil fertility remaining from the previous crop is sufficient to satisfy the needs of the PC (Renna et al., 2014).

The iodine biofortification was realized by spraying iodine on the leaves in form of potassium iodate (KIO_3, Sigma-Aldrich ACS reagent, purity 99.5%). Three different levels of iodine were compared, namely:

- 0—control: (no foliar biofortification—no iodine was added);
- FB-L (foliar biofortification—low level): the iodine concentration was 50 mg·L^{-1} (0.394 mM) for every application;
- FB-H (foliar biofortification—high level): the iodine concentration was 500 mg·L^{-1} (3.94 mM) for every application.

In total, four foliar applications were realized: the first one was realized on January 30, at the plant stage of full vegetative growth (30 cm height), while the other applications were distributed fortnightly, the latest one with the roots fully developed. For every treatment, the volume used was 1 L·m^{-2}. The irrigation was done by sprinkling.

The Harvest Index (HI) was calculated with the following formula:

$$FW\ (roots)/FW\ (roots) + FW\ \left(aerial\ biomass\right)$$

where FW = fresh weight.

Greenhouse—Crop System and Treatments

The trial was carried out at the "La Noria" experimental farm (Institute of Sciences of Food Production of the National Research Council) located in Mola di Bari (41.06214, 17.06685—Southern Italy), in a polymetacrylate non heated greenhouse with a maximum height of 4.5 m. The temperature ranged from 4° to 34°C and the percentage of relative humidity from 25 to 99% during the crop cycle (minimum/maximum respectively—data not shown).

Plants were arranged on 12 aluminum benches (length 6 m, width 0.26 m, 1% sloped) on which were positioned 20 pots for bench, each of which had a volume of 8.5 L. The distances of the pots on the rows and the distance between the rows were 0.2 m and 0.33 m, respectively. The treatments were arranged in a randomized block design, with two replications and four benches that served as guard rows, two external and two between the blocks. Every pot contained perlite as substrate (AGRILIT 3, Perlite Italiana) and, in the upper part, a peat layer of 0.5 cm that was positioned in order to ensure a uniform distribution of the watering in the first phases of seeds germination. The sowing was realized on November 18, by putting 4 to 6 seeds per hole, in four holes arranged according to the vertexes of a square. After the complete seedling emergence, at the stage of 1st–2nd true leaf, a thinning was done to have four plants per hole.

On December 27, the fertigation with nutrient solution (NS) was started. The NS, which pH and electric conductivity (EC) values were 5.7 and 3.4 dS·m^{-1}, respectively, had the following composition: N (14 mM), P (1 mM), K (6 mM), Mg (2 mM), Ca (4 mM), S (2 mM), Fe (20 μM), Mn (5 μM), Zn (2 μM), B (25 μM), Cu (0.5 μM), and Mo (0.1 μM). Fertigation was realized by using a pressure compensating emitter per pot, with a flow of 8 L·h^{-1}. The NS was managed in open cycle, and the frequency of irrigations was adjusted to maintain the drainage percentage between 30 and 50%. The experimental treatments were differentiated on March 7, by means of potassium iodate (KIO_3, Sigma-Aldrich ACS reagent, purity 99.5%). The treatments were the following:

- 0—control: (no foliar biofortification—no iodine was added);
- FB-L (foliar biofortification—low level): the iodine concentration was 50 mg·L^{-1} (0.394 mM);
- FB-H (foliar biofortification—high level): the iodine concentration was 500 mg·L^{-1} (3.94 mM);
- NS-L (NS biofortification—low level): the iodine concentration was 50 mg·L^{-1} (0.394 mM);

The foliar applications were repeated fortnightly (three applications in total—at the same plant stage of open field experiment), while the iodine into the NS was provided continuously until the harvest.

Measurements and Analysis

In the second experiment, we have analyzed the three colors of the carrots, namely, yellow, orange and purple.

The dry matter (DM) was determined after drying until constant weight in a forced-draft oven at 65°C, for at least 72 h.

The concentration of the nitrates was determined by ion chromatography (Dionex model DX120; Dionex Corporation, Sunnyvale, CA, USA) with a conductivity detector, using the pre-column IonPack AG14 and the column of separation IonPack AS14 (Signore et al., 2016).

Extraction of Inorganic Iodine

For the extraction and quantification of inorganic iodine, the roots were separated into the different colors (yellow, orange, and purple).

The inorganic iodine determination was determined using the protocol by Perring et al. (2001). Briefly, for analysis of Iodine content, 2–3 g of lyophilized carrots samples were treated with 50 mL of hot water (60°C) and stirred for 30 min at room temperature. After extraction, the samples were diluted and filtered by using Whatman filter paper followed by 0.2 μm membrane filter. The resulting solutions were used for quantification of inorganic iodine content.

Quantification of Inorganic Iodine Content

The analysis of inorganic iodine contents was determined using the spectrophotometric methods described by Perring et al. (2001). Briefly, iodate standard solution and the extracts samples (100 μg·L^{-1}) were treated with 1 mL of KSCN (0.023% m/v),

2 mL of $NH_4Fe(SO_4)_2$ (7.7% m/v) in 2.4 M HNO_3 and 2 mL of $NaNO_2$ (0.02% m/v). The solutions were mixed and incubated in water bath at 60 ± 2°C for 1 h and subsequently incubated for 10 min in a water–ice mixture in order to stop the colorimetric reaction. Each solution was read at 454 nm. The quantification of inorganic iodine in carrots was determined by interpolation with a calibration curve, previously made (0–12 μg/L; $R^2 = 0.9895$).

Statistical Analysis

The statistical analysis was performed with the Statistical Analysis System software SAS (Cary, NC, USA) using the GLM (General Linear Model) procedure for the analysis of variance.

For all the parameters, the comparison between the means point was performed by calculating the least significant difference (LSD, $P = 0.05$).

RESULTS

The biofortification did not affect any of the biometric parameters in the open field experiment (**Table 1**) as well as in greenhouse (**Table 2**). The FW of leaves and roots (**Table 2**) and the harvest index (data not shown) were influenced neither by the type of biofortification nor by the level of iodine used.

The nitrate (NO_3^-) concentration on DM basis was not influenced by the biofortification, but the treatments acted jointly with part of the plant in determine some significant differences (**Figure 1**), even if the NO_3^- concentration measured on the fresh basis was not different (data not shown). Into the roots, the NO_3^- concentration was higher in the control treatment with respect to the biofortified ones (**Figure 1**), while in the leaves the only significant difference was between FB-L and FB-H, with the latter that produced a NO_3^- concentration 112% higher with respect to FB-L (**Figure 1**). The difference in NO_3^- concentration between leaves and roots is clearly visible in the control treatment, but the biofortification treatment has flattened such difference (**Figure 1**).

The biofortification has increased the iodine concentration of the roots in both open field (51% in FB-L treatment and 194% in FB-H treatment with respect to the non-biofortified carrots—**Table 1**) and greenhouse experiment (but only when the iodine was applied by the means of the NS—**Table 2**), even if the different colors of the carrot did not produce any significant difference (data not shown).

DISCUSSION

In our experiments, the biometric parameters were not influenced by the biofortification treatments, not in open field nor in greenhouse (**Tables 1**, **2**) in agreement with the results reported by Smolen et al. (2014). Generally, high concentration of iodine may lead to a detriment of biomass even at low rates, as reported by Caffagni et al. (2011), whose iodine levels ranged from 0 to 23 mM, but this was not our case. Such result is not surprising, since carrot is reported to have a good tolerance to high levels of iodine (Hong et al., 2008). However, when the iodine was distributed via the NS, we observed a negative effect on the leaves (**Figure 2**) i.e., necrosis on the leaves, in particular in the outer margins of the older ones, without any effect on the roots. As reported by other Authors, such negative aspect is usually more pronounced into the shoot than in the "below ground" organs (Hong et al., 2008; Caffagni et al., 2011), probably because iodine is transported in the xylem rather than in the phloem (Herrett et al., 1962; Mackowiak and Grossl, 1999; Mackowiak et al., 2005). The magnitude of the injuries on the leaves depends on several factors such as the species, the chemical form of iodine used, the method of iodine application and the crop environment (open field or greenhouse) (Mackowiak et al., 2005; Weng et al., 2008a,b; Caffagni et al., 2011). In our case, this negative effect would indicate that, in a soil-less system, the iodine distributed via the NS accumulates continuously and cumulatively into the leaves tissues; therefore, the concentration of iodine that should be used within the NS has to be previously considered, by both decreasing its concentration into the NS and avoiding its application during every single fertigation.

The nitrates concentration on DM basis was not influenced by the biofortification, but the iodine concentration and the organ considered (leaved and roots) modulated the final concentration, as reported in **Figure 1**, and is clearly visible that the difference in nitrate concentration between leaves and roots has been flattened by such interaction. Such result may be explained with the existing linkage between the metabolism of IO_3^- and the reduction of NO_3^- into the plant tissue (Wong and Hung, 2001; Hung et al., 2005; Smolen et al., 2014), even if the data regarding such interaction are sometimes ambiguous (Gonda et al., 2007). Blasco et al. (2010) in and experiment with lettuce, compared three level (20, 40, and 80 $\mu mol \cdot L^{-1}$) and two forms of iodine (I^- and IO_3^-), and found that the application of I^- (as KI), at levels of at least 20 μM, reduced both NO_3^- accumulation and NR activity (NR_{act}) in leaves of lettuce. Conversely, the same levels of iodine, but in the forms of IO_3^- (as KIO_3), increased NR_{act}, but did not produce any influence on NO_3^- concentration in lettuce leaves. In our case, the concentration of NO_3^- into the roots of the biofortified treatments (**Figure 1**) was not different from that into the leaves. In the control treatment, the NR_{act} into the roots was probably involved mainly in the reduction of IO_3^- to I^- at the expense of the NO_3^- reduction process, while in the biofortified treatments the NR_{act}, which value was probably higher with respect to the control treatment, consistently with the results of Blasco et al. (2010), could cope with the reduction of both IO_3^- to I^- and NO_3^- to NO_2^-. The higher NO_3^- concentration in the leaves of the FB-H with respect to the FB-L treatment, could be explained by the level of iodine used: in fact, as reported by Blasco et al. (2010), the NR_{act} rose up to a certain concentration of iodine, then was reduced, hence inducing an higher concentration of nitrates. Moreover, the carrot accumulates more iodine into the shoot than the root (Hong et al., 2008), so this could have led to a greater suppression of NR_{act} in the leaves.

The iodine concentration of carrot roots was increased by the biofortification treatments both in open field (174, 89, and 59 $\mu g \cdot 100\,g$ of FW^{-1} for FB-H, FB-L and the control, respectively) and in greenhouse, but in this case only with the iodine applied by the means of the NS (**Table 2**), consistently with what has been reported by other authors (Dai et al., 2004; Hong et al.,

TABLE 1 | Iodine concentration in the roots, fresh and dry matter weight in leaves and roots—open field.

Biofortification	Iodine (μg·100 g FW^{-1})	Leaves		Roots	
		Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
0	59 c (±5.98)	219 (±10.20)	25.2 (±0.82)	115 (±11.71)	9.86 (±0.88)
FB-L	89 b (±10.63)	209 (±11.61)	27.6 (±2.18)	111 (±5.24)	10.13 (±0.33)
FB-H	174 a (±10.68)	217 (±1.10)	27.1 (±2.23)	109 (±6.73)	9.43 (±1.17)
Significance[a]	***	ns	ns	ns	ns

[a]*Significance of F: ns, not significant for P ≤ 0.05; ***, significant for P ≤ 0.001. Different letters indicate statistically significant differences at P = 0.05. Number of observations (replications) = 3.*

TABLE 2 | Fresh weight and dry matter percentage in leaves and roots and iodine concentration in roots—greenhouse.

Biofortification	Leaves		Roots		Iodine (μg·100 g FW^{-1})
	Fresh weight (g)	Dry matter (%)	Fresh weight (g)	Dry matter (%)	
0	258 (±50.1)	7.95 (± 7.9)	66.9 (± 21.4)	7.16 (± 0.8)	1.2 c (±0.04)
FB-L	221 (±50.7)	8.81 (± 8.8)	53.6 (± 15.7)	7.35 (± 1.2)	35.4 bc (±2.53)
FB-H	283 (±146.5)	7.94 (± 7.9)	76.2 (± 28.7)	7.04 (± 0.5)	75.1 b (±1.11)
NS-L	280 (±60.7)	9.08 (± 9.1)	88.7 (± 28.8)	8.10 (± 0.8)	896.0 a (±43.5)
Significance[a]	ns	ns	ns	ns	***

[a]*Significance of F: ns, not significant for P ≤ 0.05; ***, significant for P ≤ 0.001. Different letters indicate statistically significant differences at P = 0.05. Number of observations = 6 (2 replications and three colors).*

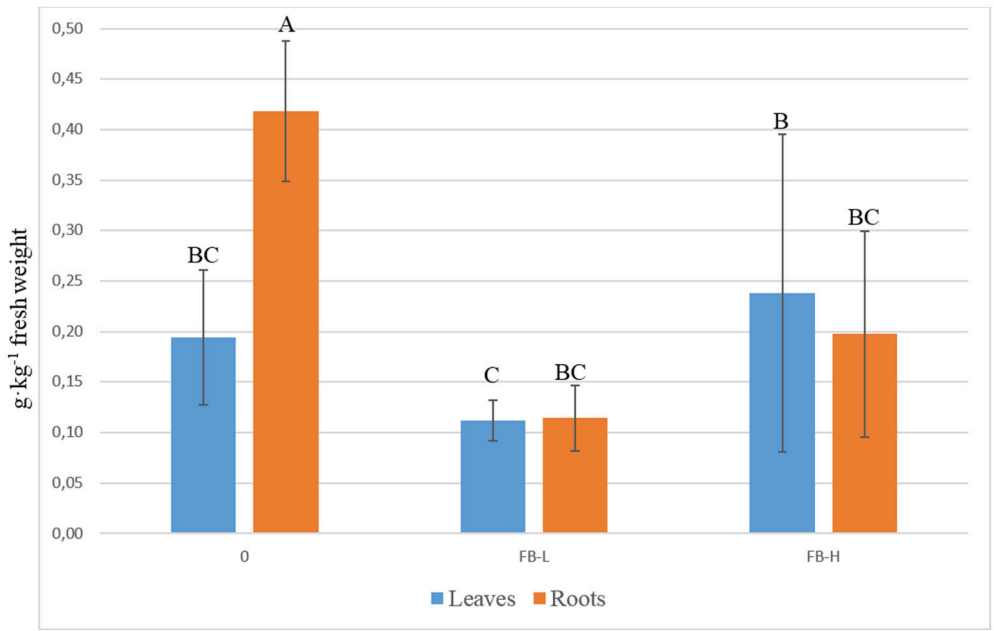

FIGURE 1 | Concentration of the nitrates according with the part of considered plant and as a function of the experimental treatment—open field. Vertical bars represent the standard deviation. Different letters indicate that mean values are significantly different, according to the LSD method $P = 0.05$.

2008; Smolen et al., 2014, 2016), even if the different colors of the carrot did not produce any significant difference (data not shown). Comparing the same levels of iodine in the FB treatments, the biofortification in the greenhouse seems to be less efficient than in open field (**Tables 1**, **2**, respectively): however, such differences were almost surely due to the number of foliar biofortification applications. Indeed, we did four iodine application in open field, and three in the greenhouse, because in the latter there was a sudden flowering of the plants due the abnormal high temperatures reached (with maximum values

FIGURE 2 | Iodine injuries on the outer margin of the leaves.

higher than 30°C starting from February—data not shown). Interestingly, the iodine concentration of the carrots from the control treatment (without biofortification) in open field, showed an iodine concentration of 59 $\mu g \cdot 100\,g\ FW^{-1}$ (**Table 1**): such concentration is surely due to the small iodine concentration contained in both the irrigation water and in the soil (15 $\mu g \cdot L^{-1}$ and 2.5 $mg \cdot kg^{-1}$, respectively—data not shown). In open field, the iodine values in the carrots would allow to satisfy, or slightly exceed, the RDA of an adult (150 $\mu g \cdot day^{-1}$—European Food Safety Authority, 2014) by consuming 100 and 200 g of fresh product for the FB-H and FB-L treatment, respectively, in agreement with the values reported by Smolen et al. (2016). This is an important point, since the carrots may be consumed either raw (they keep almost all the iodine) or cooked. The cooking process may diminish the iodine availability in biofortified foods depending on the cooking method (Winger et al., 2008; Cerretani et al., 2014). On the other side, the level of iodine reached in the NS treatment (**Table 2**) was too high for both the plants and for human nutrition purposes. That means that, in a soil-less system, the dosage of the iodized fertilizer should be strictly optimized: indeed, a high dosage may disrupt the normal plant growth and reduce the efficiency of iodized fertilizer usage (Piatkowska et al., 2016). Such aspect was highlighted by Blasco et al. (2010), who reported that a bottleneck in the biofortification process is the need to increase the iodine concentration without any adverse effect on the plant growth. From the human health point of view, the iodine level in the NS treatment could be dangerous for human health, because the iodine toxicity may cause a wide spectrum of thyroid disorders, that may range from hyperthyroidism to hypothyroidism (Comandini et al., 2013; Cerretani et al., 2014), so further research are needed to tailor the amount of iodine in the NS for carrot biofortification.

Eventually, we choose the carrot as target for iodine biofortification because increasing the content of iodine in carrot instead of other vegetables has another positive aspect: it contains high level of β-carotene, a precursor of vitamin A, that has been found to have a positive effect on thyroid function (Zimmermann, 2007). Moreover, we did consider the biofortification of local landraces of carrot because, according to Cefola et al. (2012), they have a higher nutritional value with respect to commercial ones, and the need to increase the food production at worldwide level cannot be separated from the protection of biodiversity and traditional food and practices (Caffagni et al., 2011) since, as stated by Johns and Eyzaguirre (2007), "food biofortification, in order to have a positive impact, must be complemented both with conservation and greater use of biodiversity."

CONCLUSIONS

The IDD is a major problem even in the developed countries, and biofortification with iodine is a good alternative to iodized salt to cope with such problem. Our results showed that the iodine biofortification in the carrot root can be done via foliar treatments or fertigation but, in the latter case, further studies are needed to tailor the concentration of iodine and avoiding levels that may be harmful for the plants and human health. However, the iodine concentration reached with the foliar treatments would ensure an adequate RDA by simply consuming 150 g of product fresh weight. Since we found, from our previous study, that the qualitative profile of the "Polignano carrot" is really interesting, further studies are needed to clarify the effect of iodine biofortification on β-carotene contents, antioxidant activity, total carotenoids and total phenols.

AUTHOR CONTRIBUTIONS

AS: Substantial contributions to the conception or design of the work; drafting the work; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. MR: revised the article critically; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. MD: Performed the analysis of iodine; revised the article critically; final approval of the version to be published. FS: Interpretation of data; revised the article critically; final approval of the version to be published; agreement to be accountable for

all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. PS: Substantial contributions to the conception or design of the work; analysis and interpretation of data; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

This study was supported by Regione Puglia Administration under the "Rural Development Programme (RDP) 2014–2020 - project "Biodiversity of vegetable crops in Puglia (BiodiverSO)," Measure 10, Sub measure 10.2, Operation 1 "Programme for the conservation and the valorization of the genetic resources in agriculture" and "Intervento cofinanziato dal Fondo di Sviluppo e Coesione 2007–2013, APQ Ricerca Regione Puglia – Programma regionale a sostegno della specializzazione intelligente e della sostenibilità sociale ed ambientale FutureInResearch" - project "Innovazioni di prodotto e di processo per la valorizzazione della Biodiversità Orticola pugliese (InnoBiOrt)."

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00170/full#supplementary-material

REFERENCES

Bath, S. C., Hill, S., Goenaga Infante, H., Elghul, S., Nezianya, C. J., and Rayman, M. P. (2017). Iodine concentration of milk-alternative drinks available in the UK in comparison with cows' milk. *Bri. J. Nutr.* 20, 1–8. doi: 10.1017/S0007114517002136

Blasco, B., Rios, J. J., Cervilla, L. M., Sánchez-Rodríguez, E., Rubio-Wilhelmi, M. M., Rosales, M. A., et al. (2010). Photorespiration process and nitrogen metabolism in lettuce plants (*Lactuca Sativa* L.): induced changes in response to iodine biofortification. *J. Plant Growth Reg.* 29, 477–486. doi: 10.1007/s00344-010-9159-7

Caffagni, A., Arru, L., Meriggi, P., Milc, J., Perata, P., and Pecchioni, N. (2011). Iodine fortification plant screening process and accumulation in tomato fruits and potato tubers. *Comm. Soil Sci. Plant Anal.* 42, 706–718. doi: 10.1080/00103624.2011.550372

Cefola, M., Pace, B., Renna, M., Santamaria, P., Signore, A., and Serio, F. (2012). Compositional analysis and antioxidant profile of yellow, orange and purple *Polignano carrots*. *Italian J. Food Sci.* 24, 284–291.

Cerretani, L., Comandini, P., Fumanelli, D., Scazzina, F., and Chiavaro, E. (2014). Evaluation of iodine content and stability in recipes prepared with biofortified potatoes. *Int. J. Food Sci. Nutr.* 7486, 1–6. doi: 10.3109/09637486.2014.917155

Comandini, P., Cerretani, L., Rinaldi, M., Cichelli, A., and Chiavaro, E. (2013). Stability of iodine during cooking: investigation on biofortified and not fortified vegetables. *Intl. J. Food Sci. Nutr.* 64, 857–861. doi: 10.3109/09637486.2013.798270

Dai, J. L., Zhu, Y. G., Huang, Y. Z., Zhang, M., and Song, J. L. (2006). Availability of iodide and iodate to spinach (*Spinacia Oleracea* L.) in relation to total iodine in soil solution. *Plant Soil* 289, 301–308. doi: 10.1007/s11104-006-9139-7

Dai, J. L., Zhu, Y. G., Zhang, M., and Huang, Y. Z. (2004). selecting iodine-enriched vegetables and the residual effect of iodate application to soil. *Biol. Trace Elem. Res.* 101, 265–276. doi: 10.1385/BTER:101:3:265

de Benoist, B., McLean, E., Andersson, M., and Rogers, L. (2008). Iodine deficiency in 2007: global progress since 2003. *Food Nutr. Bull.* 29, 195–202. doi: 10.1177/156482650802900305

Elia, A., and Santamaria, P. (2013). Biodiversity in vegetable crops: a heritage to save. The case of the puglia region. *Italian J. Agron.* 8, 21–34. doi: 10.4081/ija.2013.e4

European Food Safety Authority (2014). Scientific opinion on dietary reference values for iodine. *EFSA J.* 12, 1–57. doi: 10.2903/j.efsa.2014.3660

Gonda, K., Yamaguchi, H., Maruo, T., and Shinohara, Y. (2007). Effects of iodine on growth and iodine absorption of hydroponically grown tomato and spinach. *Hort. Res. Japan* 6, 223–227. doi: 10.2503/hrj.6.223

Gunnarsdottir, I., and Dahl, L. (2012). Iodine intake in human nutrition: a systematic literature review. *Food Nutr. Res.* 56, 19731. doi: 10.3402/fnr.v56i0.19731

Haldimann, M., Alt, A., Blanc, A., and Blondeau, K. (2005). Iodine content of food groups. *J. Food Comp. Anal.* 18, 461–471. doi: 10.1016/j.jfca.2004.06.003

Herrett, R. A., Hatfield, H. H., Crosby, D. G., and Vlitos, A. J. (1962). Leaf abscission induced by the iodide ion. *Plant Physiol.* 37, 358–363. doi: 10.1104/pp.37.3.358

Hong, C. L., Weng, H. X., Qin, Y. C., Yan, A. L., and Xie, L. L. (2008). Transfer of iodine from soil to vegetables by applying exogenous iodine. *Agron. Sustain. Dev.* 28, 575–583. doi: 10.1051/agro:2008033

Hung, C. C., Wong, G. T. F., and Dunstan, W. M. (2005). Iodate reduction activity in nitrate reductase extracts from marine phytoplankton. *Bull. Mar. Sci.* 76, 61–72.

Johns, T., and Eyzaguirre,. P. B. (2007). Biofortification, biodiversity and diet: a search for complementary applications against poverty and malnutrition. *Food Pol.* 32, 1–24. doi: 10.1016/j.foodpol.2006.03.014

Kiferle, C., Gonzali, S., Holwerda, H. T., Ibaceta, R. R., and Perata, P. (2013). Tomato fruits: a good target for iodine biofortification. *Front. Plant Sci*. 4:205. doi: 10.3389/fpls.2013.00205

Landini, M., Gonzali, S., and Perata, P. (2011). Iodine biofortification in tomato. *J. Plant Nutr. Soil Sci.* 174, 480–486. doi: 10.1002/jpln.201000395

Mackowiak, C. L., and Grossl, P. R. (1999). Iodate and iodide effects on iodine uptake and partitioning in rice (*Oryza Sativa* L.) grown in solution culture. *Plant Soil* 212, 135–143. doi: 10.1023/A:1004666607330

Mackowiak, C. L., Grossl, P. R., and Cook, K. L. (2005). Iodine toxicity in a plant-solution system with and without humic acid. *Plant Soil* 269, 141–50. doi: 10.1007/s11104-004-0401-6

Perring, L., Basic-Dvorzak, M., and Andrey, D. (2001). Colorimetric determination of inorganic iodine in fortified culinary products. *Analyst* 126, 985–988. doi: 10.1039/b102423j

Piatkowska, E., Kopeć, A., Biezanowska-Kopeć, R., Pysz, M., Kapusta-Duch, J., Koronowicz, A. A., et al. (2016). The impact of carrot enriched in iodine through soil fertilization on iodine concentration and selected biochemical parameters in wistar rats. *PLoS ONE* 11:e0152680. doi: 10.1371/journal.pone.0152680

Renna, M., Serio, F., Signore, A., and Santamaria, P. (2014). The yellow–purple polignano carrot (*Daucus Carota* L.): a multicoloured landrace from the puglia region (Southern Italy) at risk of genetic erosion. *Gen. Res. Crop Evol.* 61, 1611–1619. doi: 10.1007/s10722-014-0155-9

Signore, A. (2016). Mapping and sharing agro-biodiversity using open data kit and google fusion tables. *Comp. Elec. Agric.* 127, 87–91. doi: 10.1016/j.compag.2016.06.006

Signore, A., Serio, F., and Santamaria, P. (2016). A targeted management of the nutrient solution in a soilless tomato crop according to plant needs. *Front. Plant Sci.* 7:391. doi: 10.3389/fpls.2016.00391

Smolen, S., Skoczylas, Ł., Ledwozyw-Smolen, I., Rakoczy, R., Kopeć, A., Piatkowska, E., et al. (2016). Biofortification of carrot (*Daucus Carota* L.) with iodine and selenium in a field experiment. *Front. Plant Sci.* 7:730. doi: 10.3389/fpls.2016.00730

Smolen, S., Wierzbinska, J., Sady, W., Kołton, A., Wiszniewska, A., and Liszka-Skoczylas, M. (2015). Iodine biofortification with additional application of salicylic acid affects yield and selected parameters of chemical composition of tomato fruits (*Solanum Lycopersicum* L.). *Sci. Hortic.* 188, 89–96. doi: 10.1016/j.scienta.2015.03.023

Smolen, S., Sady, W., Ledwozyw-Smolen, I., Strzetelski, P., Liszka-Skoczylas, M., and Rozek, S. (2014). Quality of fresh and stored carrots depending on iodine and nitrogen fertilization. *Food Chem.* 159, 316–322. doi: 10.1016/j.foodchem.2014.03.024

Weng, H. X., Weng, J. K., Yan, A. L., Hong, C. L., Yong, W. B., and Qin, Y. C. (2008a). Increment of iodine content in vegetable plants by applying iodized fertilizer and the residual characteristics of iodine in soil. *Biol. Trace Elem. Res.* 123, 218–28.

Weng, H. X., Yan, A. L., Hong, C. L., Xie, L. L., Qin, Y. C., and Cheng, C. Q. (2008b). Uptake of different species of iodine by water spinach and its effect to growth. *Biol. Trace Elem. Res.* 124, 184–194. doi: 10.1007/s12011-008-8137-4

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets-iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

Winger, R. J., König, J., House, D. A., Konig, J., and House, D. H. (2008). Technological issues associated with iodine fortification of foods. *Trends Food Sci. Technol.* 19, 94–101. doi: 10.1016/j.tifs.2007.08.002

Wong, G. T. F., and Hung, C. C. (2001). Speciation of dissolved iodine: integrating nitrate uptake over time in the oceans. *Contin. Shelf Res.* 21, 113–128. doi: 10.1016/S0278-4343(00)00086-8

World Health Organization (2005). *Preventing Chronic Diseases: A Vital Investment*. Available online at: http://www.who.int/entity/chp/chronic_disease_report/contents/part1.pdf?ua=1

World Health Organization (2007). *Assessment of the Iodine Deficiency Disorders and Monitoring Their Elimination*. Geneva: WHO.

Zimmermann, M. B. (2007). Interactions of vitamin a and iodine deficiencies: effects on the pituitary-thyroid axis. *Int. J. Vit. Nutr. Res.* 77, 236–240. doi: 10.1024/0300-9831.77.3.236

Zimmermann, M. B. (2017). "Iodine," in *Nutrition and Health in a Developing World*, eds S. de Pee, D. Taren, and M. Bloem (Cham: Springer International Publishing), 287–295. doi: 10.1007/978-3-319-43739-2_12

Zimmermann, M. B., and Andersson, M. (2012). Update on iodine status worldwide. *Curr. Opin. Endocrinol. Diab. Obes.* 19, 382–387. doi: 10.1097/MED.0b013e328357271a

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 November 2018
doi: 10.3389/fpls.2018.01778

Recovering Tomato Landraces to Simultaneously Improve Fruit Yield and Nutritional Quality Against Salt Stress

*Isabel L. Massaretto[1,2], Irene Albaladejo[1], Eduardo Purgatto[2], Francisco B. Flores[1], Félix Plasencia[1], Jose M. Egea-Fernández[3], Maria C. Bolarin[1] and Isabel Egea[1]**

[1] Department of Stress Biology and Plant Pathology, Centro de Edafología y Biología Aplicada del Segura, CEBAS-CSIC, Murcia, Spain, [2] Department of Food Science and Experimental Nutrition, Faculty of Pharmaceutical Sciences, Food Research Center (FoRC-CEPID), University of São Paulo, São Paulo, Brazil, [3] Department of Plant Biology, University of Murcia, Murcia, Spain

OPEN ACCESS

Edited by:
Lillian Barros, Polytechnic Institute of Bragança, Portugal

Reviewed by:
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy
Francesco Di Gioia, Pennsylvania State University, United States

***Correspondence:**
Isabel Egea
iegea@cebas.csic.es

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *05 July 2018*
Accepted: *15 November 2018*
Published: *30 November 2018*

Citation:
Massaretto IL, Albaladejo I, Purgatto E, Flores FB, Plasencia F, Egea-Fernández JM, Bolarin MC and Egea I (2018) Recovering Tomato Landraces to Simultaneously Improve Fruit Yield and Nutritional Quality Against Salt Stress. Front. Plant Sci. 9:1778. doi: 10.3389/fpls.2018.01778

Salt stress generally induces important negative effects on tomato (*Solanum lycopersicum*) productivity but it may also cause a positive effect improving fruit quality, one of the greatest challenges in nowadays agriculture. Because of the genetic erosion of this horticultural species, the recovery of locally adapted landraces could play a very important role in avoiding, at least partially, production losses and simultaneously improving fruit quality. Two tomato landraces endemic of the Spanish Southeast area, characterized by the harsh climatic conditions of the Mediterranean basin, have been selected: Negro Yeste (NY) characterized by its dark-red colored fruits and Verdal (V), which fruits did not achieve the characteristic red color at ripening. Here the agronomic, physiological, and metabolic responses of these landraces were compared with the reference tomato commercial cv. Moneymaker (MM), in plants grown without salt (control) and with salt stress (100 mM NaCl) for 70 days. The higher salt tolerance of both landraces was mainly reflected in the fruit number, as NY only reduced the fruit number in salt stress by 20% whereas in MM it was reduced till 43%, and in V the fruit number even showed an increase of 33% with salt stress. An important fruit quality parameter is soluble solids content, which increases induced by salinity were significantly higher in both landraces (60 and 78% in NY and V, respectively) compared with MM (34%). Although both landraces showed a similar response in relation to the high chlorophyll accumulation detected in their fruits, the fruit metabolic profiles were very different. Increased carotenoids levels were found in NY fruits, especially lycopene in ripe fruit, and this characteristic was observed in both control and salt stress. Contrarily, the carotenoid biosynthesis pathway was disrupted in V ripe fruits, but other metabolites, such as Ca^{2+}, mannose, formate, and glutamate were accumulated. These results highlight the potential of tomato landraces to improve nutritional fruit quality and maintain fruit yield stability under salt stress.

Keywords: *Solanum lycopersicum*, traditional varieties, salt tolerance, fruit quality, metabolites, carotenoids

INTRODUCTION

Agriculture is probably facing its biggest challenge in human history due to world climate change, affecting global agricultural systems, especially in arid and semi-arid areas. In these areas salinization is a growing problem due to the frequent use of irrigation waters that contain salts. This risk will increase as population rises because cities and industry will pay for the best quality water, leaving the worst to agriculture. Therefore, development of crop plants tolerant to salt stress is vital to meet the growing food demand through sustainable agriculture when saline waters are used for irrigation. Nevertheless, a positive effect generally associated with salinity is the improvement of fruit quality (Cuartero et al., 2006). Given the predicted rise in world population fruits are expected to become the main source of secondary metabolites for millions of persons in near future. Therefore, the greatest challenge in next years will be to increase simultaneously crop production and fruit quality. The quality term is very wide and may refer to intrinsic and extrinsic characteristics, as well as to preharvest and postharvest periods (Kyriacou and Rouphael, 2018). The synthesis and accumulation of health-promoting metabolites, termed phytochemicals, depends mainly on the genetic material, although the agronomic practices and environmental factors also have an important influence on yield and quality characteristics of fruits and vegetables (Rouphael et al., 2012; Schreiner et al., 2013). Thus, salt and nutritional stresses have been used for the improvement of the nutritional quality of fruits (Colla et al., 2013; Fanciullino et al., 2014). However, to date progress has been largely limited to agronomic traits, whereas most of quality attributes, particularly those related to nutrition, have not been approached so deeply because of their complexity (Kyriacou and Rouphael, 2018; McQuinn et al., 2018).

Tomato (*Solanum lycopersicum*) is one of the most important horticultural crops worldwide (FAOSTAT, 2016). Aside from its socio-economic importance tomato has become a model species for fleshy fruits because of its agronomic and genetic features, and particularly as a rich plant source of carotenoids, vitamins, and minerals (Bergougnoux, 2014; Schwarz et al., 2014). Improving nutritional quality by enhancing the contents of bioactive compounds has become an important aspect for tomato fruit quality valorization and it has emerged as a challenge for growers who want to meet the ever-increasing demands of consumers in a highly competitive fresh market (Wu and Kubota, 2008). An important trait in breeding is improvement of individual carotenoid levels given their importance as precursors of volatiles associated with sensorial quality of tomato and as fundamental bioactive compounds in human health. Thus, β-carotene, the precursor of vitamin A, is essential for the health of eye while lycopene, the most abundant carotenoid in tomato, protects against chronic diseases and it diminishes the risk of cancer and cardiovascular diseases (Fraser and Bramley, 2004; Sharoni et al., 2012). The inability of humans to synthesize carotenoids *de novo* makes them dependent on plants as their primary source of dietary carotenoids. In a broad sense, the metabolome is what we assimilate from eating a tomato fruit that determines the nutritional value of this important crop. In this research work tomato is going to be used as a model crop since its fruit quality properties can be strongly modified by environmental conditions and, furthermore, it is the horticultural species supplementing the highest amount of metabolites in human diet given its so elevated consumption per capita (Cocaliadis et al., 2014; Liu et al., 2015).

One of the problems limiting the progress for developing tomatoes containing high levels of health-promoting compounds is genetic erosion (D'Esposito et al., 2017). Recently, Zhu et al. (2018) illustrated how breeding changed the tomato fruit metabolome. A serious consequence of biodiversity loss is the displacement of locally adapted landraces showing adaptation traits to harsh climactic conditions by genetically uniform hybrids and commercial cultivars (Frison et al., 2011). Plant breeding was long ago carried out by farmers who selected for specific adaptation traits leading to the generation of landraces. By contrast, modern plant breeding has emphasized adaptation to a wide spectrum of culture conditions, which has resulted in modern agriculture depending on a small number of cultivars for major crops. The main plant sources for food are more genetically vulnerable than ever before (Dwivedi et al., 2017). Because of their nearer genetic proximity to modern cultivars than to their wild relatives, landraces, or traditional varieties, may provide solutions for enhancing crop adaptation to abiotic stress as well as being new sources of healthy and nutritious food (Gascuel et al., 2017 and references therein). With this aim, two landraces adapted to adverse climatic conditions of the Spanish Southeast, with contrasted characteristics with respect to color and size of their fruits, were characterized at the agronomic, physiological, and metabolic levels in both control and salt stress conditions. The comparative response between the commercial tomato cv. Moneymaker and both landraces was analyzed in green and ripe fruits as well as in developed leaves close to the harvested fruit truss. We demonstrate that both landraces, exhibiting very different fruit metabolic profiles, are able to avoid, at least partially, the loss of fruit yield induced by salinity and at the same time to improve their fruit quality.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The tomato (*S. lycopersicum*) commercial cv. Moneymaker (MM) and the traditional varieties Negro Yeste (NY) and Verdal (V), collected in the semiarid area of Spanish Southeast, were used in this study. The morphological traits of the fruits (shape and size) were very different among them; MM, used as reference, is a round-shaped fruit type, whereas NY fruits are small size Pera type (cherry), and V ones are big size fruits and they have high locule number (Muchamiel type). The seeds of NY and V were supplied by the Agroecology Network of the Region of Murcia (RAERM).

Seeds were germinated in darkness, in a 2:1 (v/v) mixture of peat:perlite, at 28°C temperature and 90% of relative humidity (RH). After emergence, plants were grown in a controlled growth chamber with 16 h light/8 h darkness photoperiod, and 25°C and 50–60% of temperature and RH, respectively. A spring-summer culture was carried out in a glasshouse located at

the campus of the University of Murcia (Espinardo, Region of Murcia, Spain), which offered us tight controlled culture conditions. The temperature was programmed to daily oscillate between 15 (night) and 28°C (day) and RH was maintained at 60%. At the 4th-leaf stage (30 days after sowing) 18 plants per variety were transplanted to plastic pots containing 17 L of coco peat (Projar Group,Valencia, Spain, 8 mm maximum particle size), using a drip irrigation system, with 3 L h^{-1} drippers (**Supplementary Figure 1A**). The fertigation solution (Hoagland solution, Hoagland and Arnon, 1950) was prepared in 2,000 L tanks with local irrigation water (EC = 0.9 dSm^{-1}), and pH and EC was regularly monitored. The macronutrients salts used to prepare the nutrient solution were KNO_3, $Ca(NO_3)_2{\cdot}H_2O$, NH_4NO_3, KH_2PO_4, $MgSO_4{\cdot}7H_2O$; for micronutrients, Mn $SO_4{\cdot}5H_2O$, H_3BO_3, Cu $SO_4{\cdot}5H_2O$, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and Zn $SO_4{\cdot}7H_2O$ were used, and Fe-DPTA (6%) for Fe.

At the 8th-leaf stage, salt treatment was applied to half of the plants for 70 days, by means of fertigation solution supplemented with 100 mM NaCl (PanReac AppliChem GmbH, Darmstadt, Germany), while the other half was irrigated without salt (control condition). Salt level was selected on the basis of previous studies (Campos et al., 2016; Egea et al., 2018) and its negative effect in the plants confirmed in preliminary experiments carried out under natural conditions (Egea et al., 2017). Lixiviate was collected with a frequency of 2 days once the salt treatment has started and EC and ionic composition was determined (**Supplementary Figures 1A,B**).

Differently of plants grown in our greenhouse under natural conditions, where a previous assay was carried out with three blocks, due to changing environmental conditions throughout the greenhouse (Egea et al., 2017), homogeneous environmental conditions were maintained in the whole culture surface of this glasshouse facility, which ensured us that differences were only due to two factors: genotype and salt treatment. A complete randomized design was used with nine plants per variety for each treatment (0 and 100 mM NaCl).

Ripe fruits from 2nd to 6th truss of each plant were collected, weighed and counted to estimate the two fruit yield components, fruit number and fruit weight. For ionomic and metabolic analyses, green fruits that had already reached their final size (green-mature stage, hereinafter named green fruits) and ripe fruits at commercial stage were harvested between the fourth and fifth trusses. In addition, samples of developed leaves located between these two trusses were also collected for these analyses. Except for color analysis, performed in fresh whole fruits, all samples (leaves, green fruits, and ripe fruits) were frozen and homogenized in liquid N_2 and stored at −80°C until analysis. For each variety and treatment, three biological replicates were analyzed. In each replicate, leaves, green, or ripe fruits from three individual plants were pooled.

Total Soluble Solids and Color Measurements

For the total soluble solid (TSS) content analysis, an aliquot of the tomato fruit samples were thawed and filtered through nylon membrane filter. Then the supernatant was collected and used to measure TSS using a refractometer with automatic temperature compensation (ATAGO PR-101 digital, Tokyo, Japan) and expressed as °Brix at 20°C. For each tomato subsample three technical replicates were measured. The color of fruit surface was measured by the Hunter Lab Color system (Hunter, 1975) using a chroma-meter (Minolta CR-400, Osaka, Japan).The color coordinates a* (green-red) and b* (yellow-blue) were determined and the a*/b* ratio calculated.

Total Chlorophylls and Carotenoids

In leaf measurements were determined by the method of Arnon (1949). Two hundred milligrams of freshly frozen leaves were homogenized in 20 mL 80% acetone. Homogenates were centrifuged at 3,000 × g for 10 min in a refrigerated centrifuge at 4°C. Absorbance of the supernatant was determined at 646, 663, and 470 nm, respectively. Chlorophylls and total carotenoids concentrations (μg/mL) were calculated according to the equations described by the method of Arnon (1949):

$$\begin{aligned} Chorophyll\ a &= 12.21\ A663 - 2.81\ A646 \\ Chorophyll\ b &= 20.13\ A646 - 5.03\ A663 \\ Carotenoids &= 1000\ A470 - 3.27\ Ca - 104\ Cb \end{aligned}$$

In tomato fruits these pigments were determined by the method of Nagata and Yamashita (1992). Each freshly frozen sample (1.0 g) was homogenized with 20 mL of acetone:hexane (2:3), centrifuged at 3,000 × g for 10 min in a refrigerated centrifuge at 4°C. The optical density of the supernatant was spectrophotometrically determined at 663, 645, 505, and 453 nm. Calculations of chlorophylls and carotenoids (in mg/100 mL) were made according to the equations:

$$\begin{aligned} Chorophyll\ a &= 0.999\ A663 - 0.0989\ A645 \\ Chorophyll\ b &= 1.77\ A645 - 0.328\ A663 \\ Carotenoids &= 0.216\ A663 - 1.22\ A645 - 0.304\ A505 \\ &\quad + 0.452\ A453 \end{aligned}$$

Extraction and Analysis of Cations by ICP-OES

For the analysis of Na^+, K^+, and Ca^{2+}contents dried lyophilized tissues were milled to powder, digested during 24 h in a concentrated HNO_3: $HClO_4$ (2:1 v/v) solution and analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) in a ICAP 6500 DUO/IRIS Intrepid II XLD equipment (Thermo Scientific, Waltham, MA, USA). Measurements were carried out at the Ionomics Service of CEBAS-CSIC (Murcia, Spain).

Extraction and Analysis of Sugars and Organic Acids by ^{1}H NMR

Aliquots of frozen samples were lyophilized over 48 h and then kept at −20°C in a closed recipient with Silicagel. The extraction protocol was based on Choi et al. (2004, 2006) with slight modifications. One mL of H_2O:CH_3OH 1:1 (v/v) solution

was added to 50 mg of lyophilized material, then the mixture was vortexed for 1 min, sonicated for 1 min, and subsequently centrifuged (11,000 × g at 4°C for 20 min). The supernatant was collected in a 2 mL microtube and dried with a rotary vacuum evaporator. The dried extract was reconstituted in 800 μL of a D_2O phosphate buffer (100 mM KH_2PO_4, pH = 6) containing 0.01% of TSP (0.58 mM trimethyl silyl propionic acid sodium salt) as internal standard and vortexed for 1 min. The mixture was centrifuged (16,100 × g at 4°C for 5 min) and 600 μL of the supernatant was transferred to an NMR tube for further analysis.

All ^{1}H NMR spectra were recorded at 298 K on a Bruker AVIII HD 500 NMR spectrometer (500.13 MHz for ^{1}H) equipped with a 5 mm CPP BBO cryogenic probe (Bruker Biospin, Germany). ^{1}H spectra were referenced to TSP signal (δ = 0.00 ppm), whereas ^{13}C spectra were referenced to CH-1 resonance of α-D-glucose (δ = 93.10 ppm). For each sample, 32 scans were recorded with the following parameters: 0.126 Hz/point, pulse width (PW) = 4.0 μs (30°) and relaxation delay (RD) =1.0 s. FIDs were Fourier transformed with LB =0.5 HZ, GB =0, and PC =1.0 and peak integral was used for quantitative analysis. The whole peak intensities in every 0.02 ppm in ^{1}H NMR spectra in the range of δ 0.30–12.0 were used as variables. ^{1}H NMR spectra were manually corrected for phase and baseline distortions using TOPSPIN (v3.2, Bruker Biospin). Peak-fitting on the resulting spectra was performed using a computer algorithm associated with Chenomx NMR Suite 8.1 software to generate concentrations of primary metabolites detected in plant material (Chenomx, Edmonton, AB, Canada). The region δ = 4.67–5.15 was discarded to eliminate the effects of imperfect water presaturation. The spectral areas of all buckets were normalized to the weight of extracts employed for measurements. The intensities of the selected ^{1}H resonances due to hydro-alcoholic metabolites were measured with respect to the intensity of TSP signal used as internal standard with a concentration of 0.58 mM. Measurements were carried out at the Metabolomics Service of CEBAS-CSIC (Murcia, Spain).

Extraction and Analysis of Carotenoids by UHPLC

Extraction of carotenoids was carried out using the method based on Sérino et al. (2009). One hundred microliters of 30% NaCl (w:v) solution was added to 200 mg (fruits) or 100 mg (leaves) of freshly frozen samples. The mixture was stirred for 1 min on a vortex agitator and then 200 μL of dichlorometane was added and stirred for 1 min. Subsequently, 500 μL of hexane:ether (1:1) was added and the mixture was stirred for 1 min and centrifuged (13,000 × g at 4°C for 5 min). The supernatant was collected in a 2 mL microtube; the procedure was repeated three times and the organic phases were pooled together. The remaining hexane phase was evaporated under N_2 atmosphere. The dried carotenoid extract was reconstituted in 300 μL (fruits) or 900 μL (leaves) with the injection solvent [acetonitrile (ACN)/methanol (MeOH) 7:3, v/v]/acetone 6.7:3.3, v/v, for liquid chromatographic analysis. All sample solutions were filtered through Millex 0.2 μm nylon membrane syringe filters prior to their introduction into the UHPLC equipment (Millipore, Bedford, MA, USA).

UHPLC analyses were carried out using an Acquity I Class Ultra Performance LC system connected to a TUV detector measuring absorbance at 286 and 450 nm (Waters, Milford, MA, USA). UHPLC separations were performed on a reversed-phase column Acquity UPLC C18 BEH 130 Å, 1.7 μm, 2.1 × 100 mm (Waters), using the method described by Rivera et al. (2013). The mobile phase consisted of solvent A [ACN/MeOH 7:3 (v/v)] and solvent B (ultrapure water). The gradient used started by an isocratic 80% A: 20% B for 2 min and then performing a linear gradient up to 100% A over 1 min and maintained for 8.6 min, followed by a linear gradient down to 80% A: 20% B in 1 min and this equilibrium was held maintained for 2 min to stabilize the baseline. The column temperature was set at 32°C, the flow rate was 0.4 mL/min and the total runtime was 14.6 min including column equilibration.

Identification was carried out by comparison of retention time values and spectral properties of samples with those from authentic standards, purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and CaroteNature (Lupsingen, Switzerland), and reference spectra. Standard stock solutions of major carotenoids present in leaves and fruits of tomato plants were prepared using HPLC-grade ethanol (neoxanthin, violaxanthin, and lutein) or hexane (phytoene, β-carotene, lycopene). Before use aliquots of each stock solution were diluted in their respective HPLC-grade solvent and each concentration was determined by UV-VIS absorption at their maximum absorbance wavelengths using the extinction coefficients (ε) described by Rivera and Canela (2012). Calibration was fulfilled by dose-response curves constructed from the standard solutions. Each concentration was determined by calculating the peak area and comparing it to the corresponding calibration curve. Measurements were carried out at the Metabolomics Service of CEBAS-CSIC (Murcia, Spain).

Statistical Analysis

Experimental data are presented as mean ± standard error (SE) of three biological replicates per variety and treatment. Statistical analysis was performed by two-way analysis of variance (ANOVA) and Tukey's test was applied to establish significant differences among mean values at $P < 0.05$, using the SPSS 24.0 software package. For multivariate analysis, PCA-biplot and heatmap were performed on these data matrixes and used to ascertain the overall variability among cultivars and treatments per tissue, i.e., leaves, green, and ripe fruits. Multivariate analysis was produced using the Metaboanalyst 4.0 server (Chong et al., 2018). Firstly raw data were normalized by median, processed using generalized log transformation (log 2) and then mean-centered and divided by the square root of deviation of each variable (Pareto scaling). The univariate analysis of fold change was also performed using the Metaboanalyst 4.0 server to evaluate significant differences among accumulated metabolites in fruits of landraces compared with MM.

RESULTS

The Tomato Landraces Showed Enhanced Salt Tolerance Together With Improved Fruit Quality

The two tomato traditional varieties used in this study, Negro Yeste (NY) and Verdal (V), were initially selected because of their remarkable different fruit characteristics compared with the commercial cv. Moneymaker (MM). While NY fruits were smaller than those of MM and showed a darker red color, V fruits were of greater size and did not achieve the characteristic red color when ripened (**Figure 1A**). Although important differences were observed among the three varieties in both the number of fruits per plant as well as the average fruit weight, the two landraces were less affected by salinity than MM in the fruit number (**Figure 1B**, **Supplementary Table S1**). The relative values of fruit number in salt stress with respect to control condition were significantly lower in MM (57%), while in NY achieved a 80% and in V even became higher in salt treatment than in control condition (133%). Regarding fruit weight, however, only in V it increased significantly compared with MM. Therefore, the higher salt tolerance of both landraces is mainly reflected in the fruit number.

Total soluble solids (TSS) content is one of the most important quality parameters in tomato fruits; in fact the classification of tomato products, e.g., paste or puree, is done according to their TSS contents. Under control conditions, green fruits of both landraces displayed significantly higher TSS contents while in ripe fruits this was only observed in NY (**Figure 2A**, **Supplementary Table S1**). But the most interesting results were the significant TSS increases induced by salinity in ripe fruits of both landraces compared with MM (**Figure 2A**, **Supplementary Table S1**). Interestingly, this behavior was not due to an effect of solutes concentration because of dehydration since water contents of ripe fruits were similar in the three varieties under salinity condition (**Figure 1B**, **Supplementary Table S1**). Taken together, both landraces were able to partially avoid loss of fruit yield caused by salinity and to increase TSS content.

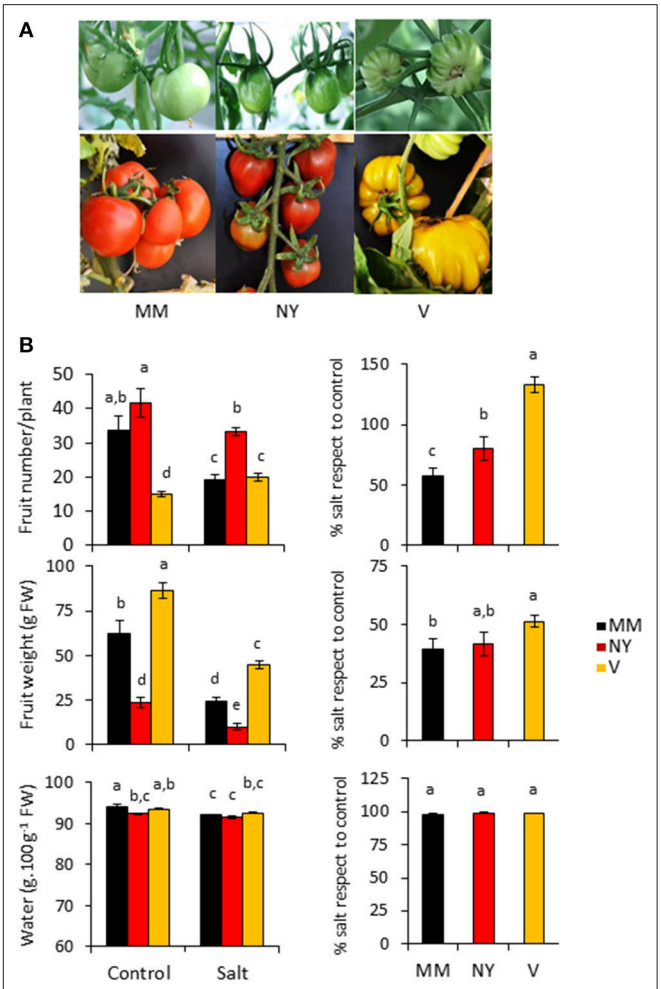

FIGURE 1 | Fruit phenotype and fruit yield parameters of Negro Yeste (NY) and Verdal (V) landraces, and commercial cv. Moneymaker (MM). **(A)** Representative images of green and ripe fruits of each variety. **(B)** Fruit number, fruit weight, and fruit water content from plants grown without stress (control) and with salt stress (100 mM NaCl during 70 days). The relative values in salt stress with respect to control condition are presented at the right-hand side of the figure. Values are expressed as mean ± SE of nine plants per variety and treatment for fruit number and weight determinations, and of three biological replicates of 10 fruits each for fruit water content determination. Different letters indicate statistically significant differences (Tukey's test; $p < 0.05$).

Fruit Color of the Tomato Traditional Varieties Reflected Differences in Pigments Contents

Ripe fruits of the two landraces have visually different colors compared with MM, and consumers consider visual color as indicator of quality (Ringeisen et al., 2014). For fruit color analysis, a*/b* coefficient was calculated from measured a* and b* color parameters (**Figure 2B**, **Supplementary Table S1**). a*/b* coefficients were negative in green fruits of the three varieties under both control and salt stress conditions, indicating the presence of the characteristic green color prior to ripening. However, a*/b* values were significant more negative in NY and V than in MM, which indicated a higher accumulation of green pigments in both landraces. The value of a*/b* turned positive in ripe fruits of MM and NY, which reflected the characteristic change of color from green to red during fruit ripening. The increase of a*/b* value was more pronounced in MM than in NY, pinpointing the fact that remaining presence of greenish tones together with red ones could be responsible of the visual red-brownish color observed in ripe NY fruits while MM fruits showed more pure red color (the highest a*/b* value). Contrarily, ripe V fruits maintained negative values of a*/b*, which is in accordance to the characteristic orange-greenish color of this landrace.

The color of tomato fruit during ripening is associated not only with carotenoids accumulation but also with chlorophylls degradation and, therefore, the differences in fruit color of the three varieties should be reflected in the pigment contents. Total contents of chlorophylls and carotenoids were measured in green and ripe fruits, and also in leaves, in order to know whether

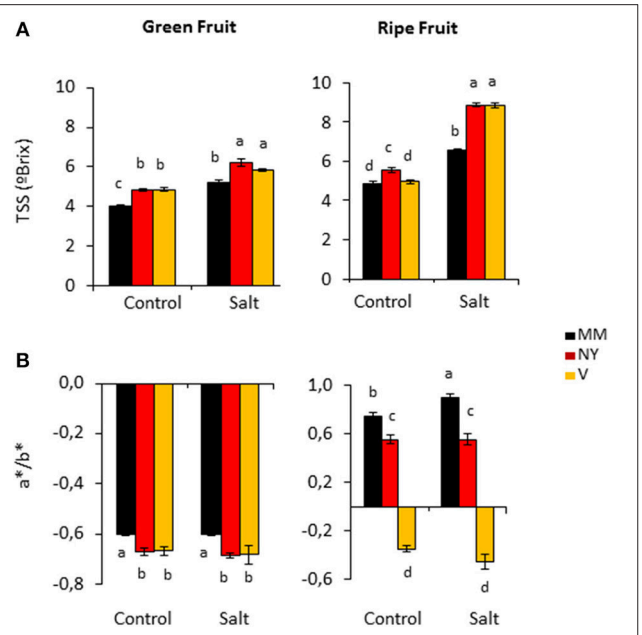

FIGURE 2 | Quality characteristics of Negro Yeste (NY), Verdal (V), and Moneymaker (MM) green and ripe fruits from plants grown without stress (control) and with salt stress (100 mM NaCl during 70 days). **(A)** Total soluble solids (TSS) and **(B)** color evaluation expressed as a*/b* ratio. Values are means ± SE of three biological replicates of ten fruits each. Different letters indicate statistically significant differences (Tukey's test; $p < 0.05$).

changes observed in fruits (sink organs) were related to changes in leaves (source organs) (**Figure 3**, **Supplementary Table S1**). While no significant differences in chlorophylls and carotenoids in MM and V leaves under control and salt conditions were detected, NY leaves showed the lowest values regarding both pigments. In green fruits both landraces showed a similar response as the chlorophylls and carotenoids contents significantly increased compared with MM, under control and, especially, under salt stress. Therefore, the high chlorophyll levels reflected the higher a*/b* value in green fruits found in both landraces. As expected, the different color of ripe fruits of both landraces is associated to different changes in the pigments contents. The red-brownish color of NY ripe fruits was due to the very high degree of carotenoids accumulation together with persistent presence of chlorophylls. V fruits presented a completely different pattern concerning its carotenoids profile when ripening, as ripe fruits had much less carotenoids than MM in both control and salt stress, maintaining almost the same levels found in green fruits, which is reflected by the orange-greenish color when ripened. Finally, it is interesting to point out the much higher increase in carotenoids induced by salt treatment in NY ripe fruits compared with MM ones, which reflects a positive facet regarding nutritional quality of these fruits.

Cations and Metabolites Contents in Leaves

In order to know whether the higher degree of salt tolerance observed in NY and V compared with MM was related to their

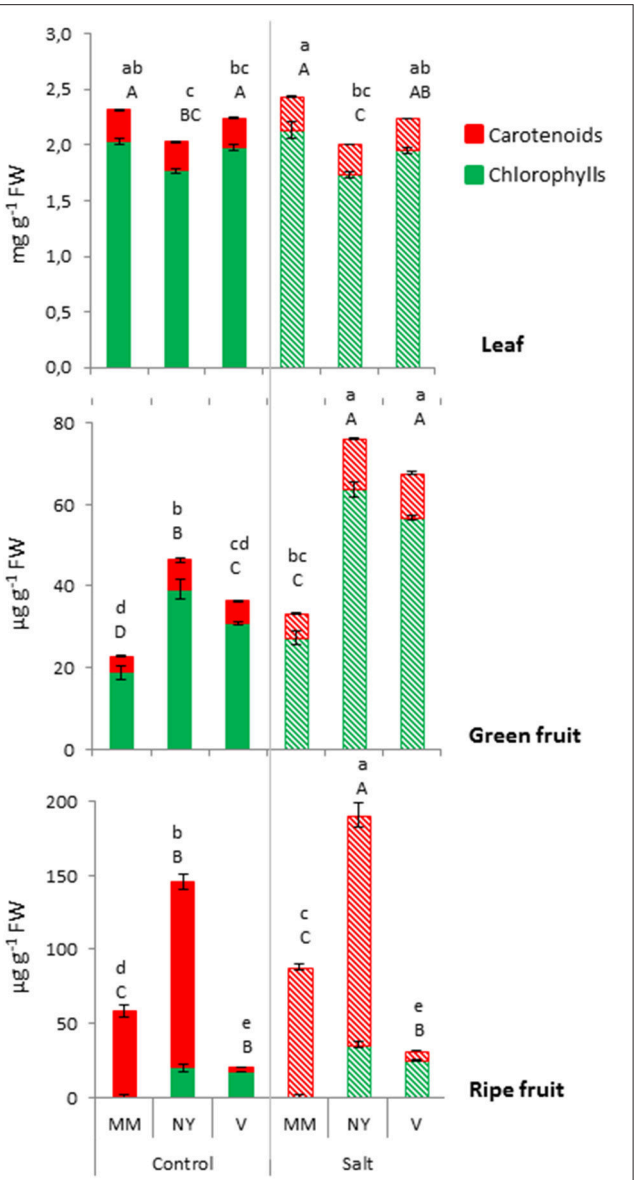

FIGURE 3 | Total chlorophylls and carotenoids contents in leaf, green, and ripe fruits from Moneymaker (MM), Negro Yeste (NY), and Verdal (V) plants grown without stress (control) and with salt stress (100 mM NaCl during 70 days). Values are means ± SE of three biological replicates of ten fruits each. Different lowercase letters indicate significant differences in total carotenoids content while different capital case letters indicate significant differences in total chlorophylls content (Tukey's test; $p < 0.05$).

different ability for ion regulation, Na^+, K^+, and Ca^{2+} contents were analyzed in leaves from control and salt-treated plants for 70 days (**Table 1**, **Supplementary Table S2**). The K^+/Na^+, and Ca^{2+}/Na^+ ratios were also calculated, since high values of these parameters are related to salt tolerance in tomato. Under control condition V presented higher K^+ content and NY higher Ca^{2+}content than MM, but the most interesting result was the significantly lower Na^+ accumulation induced by salinity in both landraces. V presented the highest K^+/Na^+, and Ca^{2+}/Na^+ ratios

TABLE 1 | Contents of Na^+, K^+, and Ca^{2+} cations and K^+/Na^+ and Ca^{2+}/Na^+ ratio values in leaf, green, and ripe fruit from plants of cv. Moneymaker and traditional varieties Negro Yeste and Verdal grown under control and salt stress conditions (100 mM NaCl during 70 days).

Cation	Moneymaker		Negro yeste		Verdal	
	Control	Salt	Control	Salt	Control	Salt
A) LEAF						
Na^+	227 ± 38^{d}	1417 ± 58^{a}	130 ± 10^{d}	910 ± 13^{b}	121 ± 2^{d}	519 ± 8^{c}
K^+	2816 ± 42^{b}	1150 ± 46^{d}	2157 ± 160^{c}	1504 ± 22^{d}	3680 ± 63^{a}	2821 ± 40^{b}
Ca^{2+}	3152 ± 52^{b,c}	3153 ± 126^{b,c}	4160 ± 300^{a}	3615 ± 33^{a,b}	2887 ± 41^{c}	3238 ± 51^{b,c}
K^+/Na^+	12.40 ± 0.02^{c}	0.81 ± 0.01^{f}	16.65 ± 0.06^{b}	1.65 ± 0.01^{e}	30.39 ± 0.07^{a}	5.44 ± 0.01^{d}
Ca^{2+}/Na^+	13.85 ± 0.01^{c}	2.22 ± 0.01^{f}	32.12 ± 0.20^{a}	3.97 ± 0.02^{e}	23.84 ± 0.02^{b}	6.24 ± 0.01^{d}
B) GREEN FRUIT						
Na^+	44.78 ± 4.44^{d}	267.42 ± 4.77^{a}	32.64 ± 0.16^{d}	181.57 ± 3.01^{c}	30.29 ± 2.07^{d}	207.65 ± 9.93^{b}
K^+	3058 ± 176^{b}	4413 ± 53^{a}	2979 ± 195^{b}	4067 ± 338^{a}	3078 ± 60^{b}	4536 ± 112^{a}
Ca^{2+}	70.18 ± 10.21^{a,b}	40.00 ± 4.50^{c}	42.86 ± 6.76^{c}	50.46 ± 8.12^{b,c}	90.84 ± 9.07^{a}	80.95 ± 4.13^{a,b}
K^+/Na^+	69.20 ± 5.23^{b}	16.52 ± 0.49^{d}	91.30 ± 6.26^{a}	22.36 ± 1.54^{c}	102.78 ± 8.69^{a}	21.92 ± 0.99^{c}
Ca^{2+}/Na^+	1.57 ± 0.16^{b}	0.15 ± 0.02^{c}	1.31 ± 0.21^{b}	0.28 ± 0.04^{c}	3.06 ± 0.45^{a}	0.39 ± 0.03^{c}
C) RIPE FRUIT						
Na^+	23.73 ± 2.16^{c}	146.37 ± 8.94^{a}	36.68 ± 1.04^{c}	124.01 ± 7.51^{a,b}	23.03 ± 1.87^{c}	103.65 ± 3.88^{b}
K^+	2349 ± 340^{a}	3161 ± 146^{a}	3015 ± 88^{a}	2510 ± 104^{a}	2596 ± 167^{a}	2537 ± 107^{a}
Ca^{2+}	30.72 ± 0.73^{c,d}	35.78 ± 1.62^{c}	55.50 ± 2.07^{b}	23.77 ± 1.48^{d}	76.76 ± 5.22^{a}	52.93 ± 1.70^{b}
K^+/Na^+	98.19 ± 6.52^{a,b}	21.63 ± 0.32^{c}	82.21 ± 0.08^{b}	20.29 ± 0.52^{c}	113.52 ± 6.95^{a}	24.50 ± 0.83^{c}
Ca^{2+}/Na^+	1.32 ± 0.16^{b}	0.25 ± 0.02^{c}	1.52 ± 0.10^{b}	0.20 ± 0.01^{c}	3.37 ± 0.31^{a}	0.51 ± 0.03^{c}

Cation contents are presented as μg g^{-1} fresh weigh. Values are the mean of three biological replicates ± standard error. Different letters in the line indicate statistically significant differences (ANOVA, Tuckey's test; $p < 0.05$).

followed by NY, while MM showed the lowest values under salt stress.

Since metabolites may be translocated from leaves (source organs) to fruits (sink organs), the major primary metabolites, sugars, and organic acids, as well as the main carotenoids were analyzed in leaves (**Table 2**, **Supplementary Table S2**) and significant changes among varieties were observed in some of them. Sucrose accumulation was significantly higher in NY, both in control and salt conditions, while similar levels were found in MM and V. In order to analyze differences among varieties, a PCA-biplot on the whole dataset, including sugars, organic acids, pigments, and cations was performed (**Figure 4A**), and a heatmap analysis illustrating the variation in the relative concentration of each metabolite for the three varieties in control and salt-treated plants (**Figure 4B**). Under control condition, relative changes are similar enough among varieties since they are displayed very close in the PC1, which accounts for 40% of the total data variance. Under salt stress, NY was clearly separated from MM and V in PC1, mainly due to its lower contents of chlorophylls and carotenoids, and MM was separated from NY and V in PC2 (25% of the total variance), where the higher contents of glucose and fructose in both landraces were the main drivers for their separation from MM. Finally, it is interesting to point out the antagonism between Na^+ and K^+, as showed by their opposite arrows in the PCA-biplot.

Cations and Metabolites Contents in Green and Ripe Fruits

With regard to changes induced by salinity in cation contents in fruit, both landraces accumulated less Na^+ than MM in green fruits, as was previously observed in leaves (**Table 1**, **Supplementary Table S2**). It is important to highlight the similar responses in leaves and green fruits of both landraces in spite of the much lower Na^+ levels attained in fruits compared with leaves. Regarding other cations, it is also noteworthy the high Ca^{2+} content found in V, especially under salt stress. Moreover, the higher Ca^{2+} levels were also maintained in V ripe fruits and, consequently, high Ca^{2+} /Na^+ ratio values were determined in fruits from this landrace.

In green fruits there were remarkable differences among varieties in the primary (sugars and organic acids) and secondary (carotenoids) metabolic profiles (**Table 2**, **Supplementary Table S2**). The PCA-biplot clearly separated the three varieties, where 50 and 20% of the total data variance was accounted for PC1 and PC2, respectively (**Figure 5A**). Na^+ and metabolites such as sucrose, carotenoids, and chlorophylls greatly contributed to separate the samples by the PC1, while succinate, malate, formate, and Ca^{2+} were important compounds for the dispersion of the samples by the PC2. Interestingly, salt stress induced a remarkable effect on the metabolic composition of the three varieties by increasing the compounds contents but keeping their different metabolic signatures, as observed from heatmap analysis (**Figure 5B**). Green NY fruits presented higher contents of sucrose, citrate, and total chlorophyll in both conditions and succinate in control than MM. The biosynthetic pathway of carotenoids seems to be up-regulated in NY green tomatoes, since the phytoene content is two-fold higher, resulting in higher contents of lutein and β-carotene in this variety compared with MM (**Table 2**, **Supplementary Table S2**). V green fruits also produced sucrose, total chlorophyll, and carotenoids at higher levels compared with MM, but contrary

TABLE 2 | Metabolites contents in leaf, green, and ripe fruit from plants of cv. Moneymaker and traditional varieties Negro Yeste and Verdal grown under control and salt stress conditions (100 mM NaCl during 70 days).

Metabolite	Moneymaker		Negro yeste		Verdal	
	Control	Salt	Control	Salt	Control	Salt
A) LEAF						
Sucrose (mg g^{-1} fw)	0.44 ± 0.01^{c}	0.49 ± 0.01^{b}	0.64 ± 0.01^{a}	0.64 ± 0.02^{a}	$0.46 \pm 0.01^{b,c}$	$0.46 \pm 0.01^{b,c}$
Fructose (mg g^{-1} fw)	0.46 ± 0.03^{c}	0.38 ± 0.01^{c}	0.77 ± 0.01^{a}	0.66 ± 0.02^{b}	$0.67 \pm 0.04^{a,b}$	0.64 ± 0.02^{b}
Glucose (mg g^{-1} fw)	0.29 ± 0.01^{c}	0.17 ± 0.01^{d}	0.46 ± 0.01^{a}	$0.40 \pm 0.02^{a,b}$	$0.43 \pm 0.02^{a,b}$	0.40 ± 0.01^{b}
Raffinose (mg g^{-1} fw)	$0.27 \pm 0.02^{a,b,c}$	0.40 ± 0.05^{a}	$0.37 \pm 0.05^{a,b}$	0.18 ± 0.02^{c}	0.39 ± 0.06^{a}	$0.23 \pm 0.05^{b,c}$
Citrate	39.49 ± 0.56^{b}	39.55 ± 3.01^{b}	62.78 ± 2.88^{a}	31.06 ± 3.11^{b}	39.10 ± 1.07^{b}	32.55 ± 2.57^{b}
Succinate	2.94 ± 0.40^{b}	2.46 ± 0.33^{c}	2.79 ± 0.12^{b}	$6.62 \pm 0.25^{a,b}$	$7.01 \pm 0.58^{a,b}$	7.74 ± 1.94^{a}
Fumarate	1.25 ± 0.23^{a}	0.92 ± 0.23^{a}	0.96 ± 0.06^{a}	1.22 ± 0.10^{a}	0.77 ± 0.01^{a}	1.10 ± 0.18^{a}
Malate	$68.56 \pm 1.07^{a,b}$	$69.58 \pm 2.13^{a,b}$	78.64 ± 5.27^{a}	$60.82 \pm 2.95^{b,c}$	50.36 ± 0.88^{c}	$71.36 \pm 2.82^{a,b}$
Formate	8.55 ± 0.36^{a}	5.89 ± 0.13^{b}	$7.15 \pm 0.3^{a,b}$	1.7 ± 0.1^{d}	7.6 ± 0.2^{a}	3.5 ± 0.3^{c}
Glutamate	77.5 ± 1.8^{d}	$109.9 \pm 3.4^{b,c}$	$89.6 \pm 2.9^{c,d}$	85.6 ± 7.9^{d}	116.6 ± 4.2^{b}	238.4 ± 2.8^{a}
Formate/succinate	3.03 ± 0.46^{a}	2.48 ± 0.34^{a}	2.57 ± 0.21^{a}	0.11 ± 0.02^{c}	1.10 ± 0.12^{b}	$0.51 \pm 0.14^{b,c}$
Glutamate/succinate	27.29 ± 3.29^{b}	46.30 ± 5.99^{a}	$32.23 \pm 2.13^{a,b}$	12.88 ± 0.71^{c}	16.80 ± 1.06^{c}	$36.83 \pm 12.08^{a,b}$
β-carotene	25.99 ± 0.69^{a}	26.92 ± 0.69^{a}	$22.01 \pm 0.69^{a,b}$	17.25 ± 0.43^{b}	28.52 ± 1.52^{a}	28.98 ± 1.47^{a}
Violaxanthin	11.56 ± 0.86^{a}	$9.45 \pm 0.98^{a,b}$	8.50 ± 0.84^{b}	3.26 ± 0.26^{c}	$9.54 \pm 0.69^{a,b}$	8.22 ± 0.29^{b}
Neoxanthin	8.80 ± 0.69^{a}	8.49 ± 0.91^{a}	$6.60 \pm 0.70^{a,b}$	4.52 ± 0.21^{b}	8.10 ± 0.28^{a}	8.54 ± 0.55^{a}
Lutein	17.05 ± 0.65^{b}	$18.92 \pm 0.65^{a,b}$	14.88 ± 0.65^{b}	11.78 ± 0.45^{c}	22.01 ± 1.23^{a}	22.88 ± 1.32^{a}
Chlorophyll a (mg g^{-1} fw)	$1.49 \pm 0.02^{a,b}$	1.54 ± 0.04^{a}	1.33 ± 0.01^{b}	1.30 ± 0.02^{b}	$1.46 \pm 0.01^{a,b}$	$1.38 \pm 0.02^{b,c}$
Chlorophyll b (mg g^{-1} fw)	0.54 ± 0.01^{a}	0.60 ± 0.03^{a}	0.44 ± 0.01^{b}	0.43 ± 0.01^{b}	$0.52 \pm 0.02^{a,b}$	0.57 ± 0.01^{a}
Chlorophyll:Carotenoid	7.23 ± 0.18^{a}	7.17 ± 0.16^{a}	$6.91 \pm 0.04^{a,b}$	6.42 ± 0.05^{b}	7.49 ± 0.27^{a}	$6.79 \pm 0.07^{a,b}$
B) GREEN FRUIT						
Sucrose (mg g^{-1} fw)	0.12 ± 0.01^{c}	0.78 ± 0.04^{b}	$0.36 \pm 0.04^{b,c}$	1.49 ± 0.11^{a}	$0.44 \pm 0.05^{b,c}$	1.85 ± 0.20^{a}
Fructose (mg g^{-1} fw)	8.5 ± 0.8^{a}	8.0 ± 0.4^{a}	8.9 ± 0.2^{a}	8.9 ± 0.2^{a}	7.5 ± 0.3^{a}	7.0 ± 0.8^{a}
Glucose (mg g^{-1} fw)	$10.52 \pm 1.28^{a,b,c}$	$10.73 \pm 0.49^{a,b,c}$	$11.94 \pm 0.40^{a,b}$	12.14 ± 0.16^{a}	$8.36 \pm 0.26^{b,c}$	7.83 ± 1.18^{c}
Mannose	11.74 ± 1.33^{b}	15.69 ± 0.87^{b}	14.79 ± 0.34^{b}	17.07 ± 0.62^{b}	12.37 ± 0.50^{b}	32.67 ± 3.07^{a}
UDP-glucose	34.66 ± 4.86^{b}	35.66 ± 2.96^{b}	40.48 ± 2.07^{b}	40.14 ± 1.89^{b}	$46.78 \pm 1.56^{a,b}$	54.83 ± 3.99^{a}
Citrate (mg g^{-1} fw)	0.71 ± 0.06^{c}	$0.81 \pm 0.05^{b,c}$	$1.04 \pm 0.05^{a,b}$	1.19 ± 0.08^{a}	0.51 ± 0.05^{d}	0.91 ± 0.01^{b}
Succinate	41.27 ± 20.97^{c}	138.32 ± 6.22^{a}	89.07 ± 4.31^{b}	126.59 ± 4.81^{a}	37.66 ± 0.99^{d}	45.28 ± 9.92^{c}
Fumarate	5.44 ± 0.37^{a}	$3.47 \pm 0.83^{a,b}$	5.92 ± 0.17^{a}	$4.43 \pm 0.12^{a,b}$	3.26 ± 0.54^{b}	$3.78 \pm 0.71^{a,b}$
Malate (mg g^{-1} fw)	$1.70 \pm 0.14^{a,b}$	1.90 ± 0.05^{a}	$1.69 \pm 0.05^{a,b}$	$1.40 \pm 0.10^{b,c}$	$0.98 \pm 0.06^{c,d}$	0.96 ± 0.11^{d}
Formate	1.8 ± 0.4^{b}	$2.3 \pm 0.4^{a,b}$	1.3 ± 0.1^{b}	1.4 ± 0.1^{b}	$2.2 \pm 0.3^{a,b}$	2.8 ± 0.3^{a}
Glutamate (mg g^{-1} fw)	0.17 ± 0.01^{c}	0.29 ± 0.02^{b}	0.19 ± 0.01^{c}	0.33 ± 0.03^{b}	0.24 ± 0.01^{b}	0.57 ± 0.1^{a}
Formate/succinate	0.06 ± 0.02^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.01 ± 0.00^{b}	0.06 ± 0.01^{a}	0.07 ± 0.02^{a}
Glutamate/succinate	6.25 ± 2.22^{b}	2.07 ± 0.13^{c}	2.14 ± 0.05^{c}	2.59 ± 0.20^{c}	6.39 ± 0.35^{b}	12.64 ± 0.28^{a}
Phytoene	2.02 ± 0.10^{c}	2.69 ± 0.29^{c}	$4.96 \pm 0.25^{a,b}$	6.18 ± 0.14^{a}	2.71 ± 0.16^{c}	4.63 ± 0.39^{b}
β-carotene	1.21 ± 0.04^{c}	1.22 ± 0.01^{c}	1.39 ± 0.12^{b}	2.01 ± 0.12^{a}	1.65 ± 0.19^{b}	$1.78 \pm 0.17^{a,b}$
Lutein	0.66 ± 0.02^{c}	$1.10 \pm 0.08^{b,c}$	1.49 ± 0.15^{b}	2.51 ± 0.28^{a}	0.43 ± 0.04^{d}	$2.14 \pm 0.17^{a,b}$
Chlorophyll a	14.14 ± 1.24^{e}	20.69 ± 1.20^{d}	28.75 ± 1.80^{c}	46.99 ± 1.12^{a}	22.49 ± 0.37^{d}	41.31 ± 0.48^{b}
Chlorophyll b	4.75 ± 0.48^{d}	$6.59 \pm 0.43^{c,d}$	10.39 ± 0.69^{b}	16.74 ± 0.69^{a}	$8.28 \pm 0.02^{b,c}$	15.44 ± 0.14^{a}
Chlorophyll:Carotenoid	4.80 ± 0.25^{a}	4.57 ± 0.41^{a}	5.45 ± 0.10^{a}	5.11 ± 0.13^{a}	5.59 ± 0.09^{a}	5.17 ± 0.14^{a}
C) RIPE FRUIT						
Sucrose (mg g^{-1} fw)	ND	0.10 ± 0.01^{c}	0.05 ± 0.01^{c}	0.30 ± 0.05^{b}	$0.16 \pm 0.07^{b,c}$	0.75 ± 0.10^{a}
Fructose (mg g^{-1} fw)	7.71 ± 0.54^{c}	$9.08 \pm 0.23^{b,c}$	$9.81 \pm 0.37^{a,b}$	10.91 ± 0.32^{a}	$8.28 \pm 0.28^{b,c}$	$9.49 \pm 0.21^{a,b}$
Glucose (mg g^{-1} fw)	$10.67 \pm 1.04^{c,d}$	$13.37 \pm 0.61^{a,b}$	$11.65 \pm 0.38^{b,c}$	14.92 ± 0.36^{a}	8.95 ± 0.25^{d}	$12.17 \pm 0.25^{b,c}$
Mannose	28.16 ± 2.92^{b}	35.90 ± 6.14^{b}	37.04 ± 3.64^{b}	26.13 ± 3.93^{b}	$44.07 \pm 2.37^{a,b}$	66.79 ± 9.02^{a}
UDP-glucose	$25.60 \pm 3.62^{a,b}$	$22.95 \pm 0.65^{a,b}$	$23.47 \pm 0.75^{a,b}$	17.24 ± 1.62^{b}	$26.89 \pm 4.77^{a,b}$	34.23 ± 6.23^{a}
Citrate (mg g^{-1} fw)	0.76 ± 0.19^{b}	1.27 ± 0.05^{a}	1.25 ± 0.09^{a}	$0.99 \pm 0.15^{a,b}$	0.73 ± 0.07^{b}	0.46 ± 0.03^{c}
Succinate	89.11 ± 8.59^{b}	114.10 ± 9.07^{a}	123.59 ± 9.22^{a}	$66.58 \pm 7.53^{b,c}$	40.62 ± 8.19^{c}	29.30 ± 5.78^{c}

(Continued)

TABLE 2 | Continued

Metabolite	Moneymaker		Negro yeste		Verdal	
	Control	Salt	Control	Salt	Control	Salt
Fumarate	4.00 ± 0.38^{a}	$2.36 \pm 0.84^{a,b}$	$1.83 \pm 0.43^{a,b}$	1.00 ± 0.182^{b}	0.45 ± 0.04^{c}	0.94 ± 0.26^{b}
Malate (mg g^{-1} fw)	1.11 ± 0.20^{b}	1.06 ± 0.12^{b}	1.72 ± 0.07^{a}	1.23 ± 0.10^{b}	0.64 ± 0.02^{c}	0.31 ± 0.04^{d}
Formate	1.31 ± 0.05^{c}	1.70 ± 0.18^{c}	1.45 ± 0.05^{c}	1.17 ± 0.02^{c}	8.75 ± 1.62^{b}	25.05 ± 1.18^{a}
Glutamate (mg g^{-1} fw)	1.20 ± 0.15^{b}	$1.41 \pm 0.05^{a,b}$	$1.68 \pm 0.14^{a,b}$	$1.81 \pm 0.14^{a,b}$	$2.17 \pm 0.30^{a,b}$	2.32 ± 0.32^{a}
Formate/succinate	0.02 ± 0.00^{c}	0.02 ± 0.00^{c}	0.01 ± 0.00^{c}	0.02 ± 0.00^{c}	0.30 ± 0.20^{b}	0.93 ± 0.20^{a}
Glutamate/succinate	14.03 ± 1.30^{d}	12.93 ± 1.84^{d}	13.68 ± 0.31^{d}	31.22 ± 7.92^{c}	63.18 ± 16.83^{b}	82.16 ± 11.95^{a}
Phytoene	5.30 ± 0.36^{b}	7.04 ± 0.18^{a}	1.73 ± 0.11^{d}	3.35 ± 0.12^{c}	1.32 ± 0.17^{d}	1.35 ± 0.02^{d}
β-carotene	3.14 ± 0.10^{b}	2.74 ± 0.19^{b}	6.22 ± 0.18^{a}	6.02 ± 0.15^{a}	1.01 ± 0.12^{c}	1.06 ± 0.07^{c}
Lycopene	50.12 ± 4.20^{d}	78.85 ± 2.24^{c}	107.19 ± 4.41^{b}	134.20 ± 8.15^{a}	ND	ND
Lutein	0.25 ± 0.01^{c}	0.36 ± 0.05^{c}	1.36 ± 0.07^{b}	2.12 ± 0.21^{a}	0.35 ± 0.04^{c}	0.65 ± 0.08^{c}
Chlorophyll a	ND	ND	15.96 ± 2.06^{b}	27.48 ± 2.16^{a}	12.05 ± 0.56^{b}	18.01 ± 2.00^{b}
Chlorophyll b	ND	ND	4.21 ± 0.55^{c}	8.13 ± 0.68^{a}	$5.11 \pm 0.18^{b,c}$	$6.76 \pm 0.52^{a,b}$
Chlorophyll:Carotenoid	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.11 ± 0.02^{c}	0.17 ± 0.02^{c}	5.05 ± 0.21^{a}	3.48 ± 0.36^{b}

Metabolites contents are presented as μg g^{-1} fresh weigh (fw) unless otherwise stated in the table. Values are the mean of three biological replicates ± standard error. Different letters in the line indicate statistically significant differences (ANOVA, Tuckey's test; $p < 0.05$). ND, not detected.

to NY, the contents of organic acids citrate, malate, fumarate, and succinate were lower than in MM, particularly in control. The most interesting metabolic changes induced by salt stress in V green fruits were the increased levels of mannose and glutamate.

Ripe tomato fruit samples were displayed strongly separated on the PCA-biplot according to variety, which accounted for 50 and 26% of the total variance explained by PC1 and PC2, respectively (**Figure 6A**). It was observed that salt-treated samples remain close to their respective controls, indicating that the compounds profiles of MM, NY, and V seem to be more associated with genotype-specific variations than with those triggered by salt stress, in contrast with the salt response observed in green fruits. Basically, the higher contents of sucrose and chlorophylls in landraces were the main traits contributing to separate them from MM. Regarding carotenoids, lycopene, β-carotene, and lutein were the main metabolites increasing in NY compared with MM, especially under salt stress (**Figure 6B**, **Table 2**, **Supplementary Table S2**). However, the V ripe fruit pattern was very different, as only lutein content was similar to that found in MM, while β-carotene and phytoene were present in much lower concentrations and lycopene was not detected. But even more important differences were found when comparing V ripe fruits with MM ones; compounds from the TCA cycle as citrate, succinate and malate were sharply reduced while very high levels of formate were induced by salt stress, as observed also by the high value of the formate/succinate ratio attained in ripe fruits of this landrace (**Table 2**, **Supplementary Table S2)**. It is also interesting to remark the high degree of accumulation of mannose detected in V ripe fruits, especially under salt stress. In sum, the metabolic composition of ripe fruits greatly differs between the two landraces and differences are also found when each landrace is compared with MM.

DISCUSSION

The Tomato Traditional Varieties Improved Cations Homeostasis and Increased Sucrose and Chlorophylls Contents in Fruits

In order to identify locally adapted traditional tomato varieties able to exhibit higher accumulation of compounds that positively influence nutritional quality and at the same time uphold fruit development under salt stress conditions, we have selected two landraces with very different fruit characteristics, showing lower reduction (NY) or even increased (V) fruit number under salinity compared with MM (**Figure 1B**). Although it is very difficult to elucidate which biological processes control plant and fruit growth and hence fruit yield (Sonnewald and Fernie, 2018), the higher salt tolerance of both landraces compared with MM was associated to increased K^+/Na^+ ratio values in leaves (**Table 1**), a physiological trait directly related with salt tolerance in tomato (García-Abellan et al., 2014). Interestingly, in spite of the much lower Na^+ content achieved in fruits compared with leaves, the K^+/ Na^+ ratios in green fruits were maintained also at higher values in landraces than in MM. The Ca^{2+} changes observed may reflect how genotype and salinity affect transport via xylem from source leaves to sink fruits, as Ca^{2+} is the only ion transported to fruits mainly through the xylem (Gilliham et al., 2011). It could be observed in NY leaves a high Ca^{2+} level while in green fruit this is much reduced and differences between control and salt stress disappeared (**Table 1**). But the most significant changes detected regarding Ca^{2+} levels are the increases in V green and ripe fruits, which is of major interest from the point of view not only of plant stress response, as salt tolerance is associated to maintenance of Ca^{2+} homeostasis (Egea et al., 2018), but also from the nutritional quality perspective as this cation is very important in a healthy human diet. In addition, from a

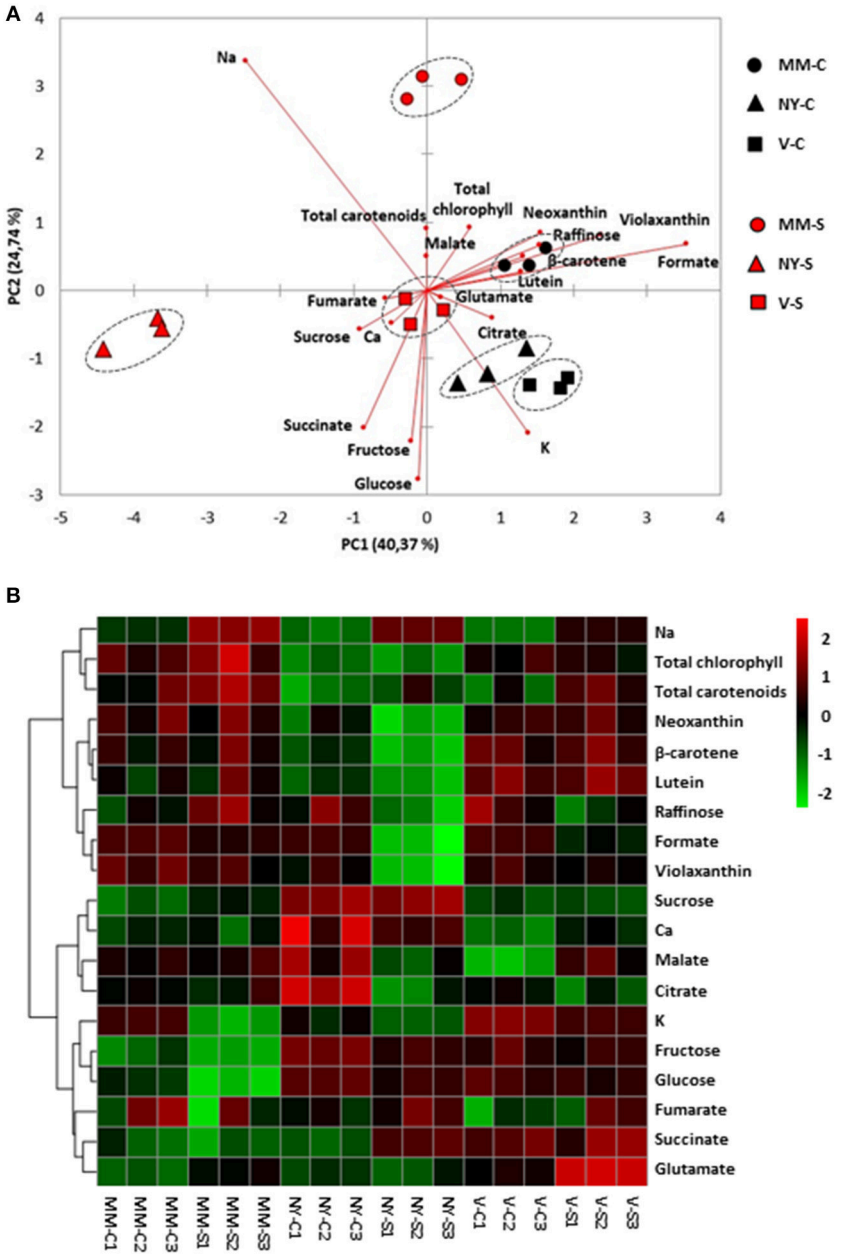

FIGURE 4 | Relative metabolites and cations contents in leaves of Moneymaker (MM), Negro Yeste (NY), and Verdal (V) from plants grown under control and salt stress (100 mM NaCl during 70 days) conditions. **(A)** Non-supervised principal component analysis (PCA-biplot) and **(B)** heatmap analysis representing the major sources of variability. Color scale represents the variation in the relative concentration of compounds, from high (red) to low (green) contents.

point of view of fruit quality, both landraces had a common characteristic: the similar TSS contents, which were significantly higher than MM, especially under salt stress. Moreover, this rising TSS content is not caused by a dehydration effect but it seems a *per se* accumulation of reducing sugars in the fruits (**Figures 1B**, **2A**).

A key factor of fruit quality is the metabolic composition of this sink organ (Osorio et al., 2014), where an important portion of metabolites are imported from source leaves. However, we observed that the metabolic profiles were more specific of variety (genotype factor) in fruits than in leaves (**Figure 4** compared with **Figures 5**, **6**), which seems to indicate that final fruit quality is mainly the result of metabolic changes in fruits rather than in leaves. Moreover, it is in green fruits where the metabolite profiles of the three varieties were clearly modulated by salt stress. Thus, considering sucrose, the main carbon link between source and sink organs, it has been observed just a major constitutive level in NY leaves but no changes with salt stress in any of the

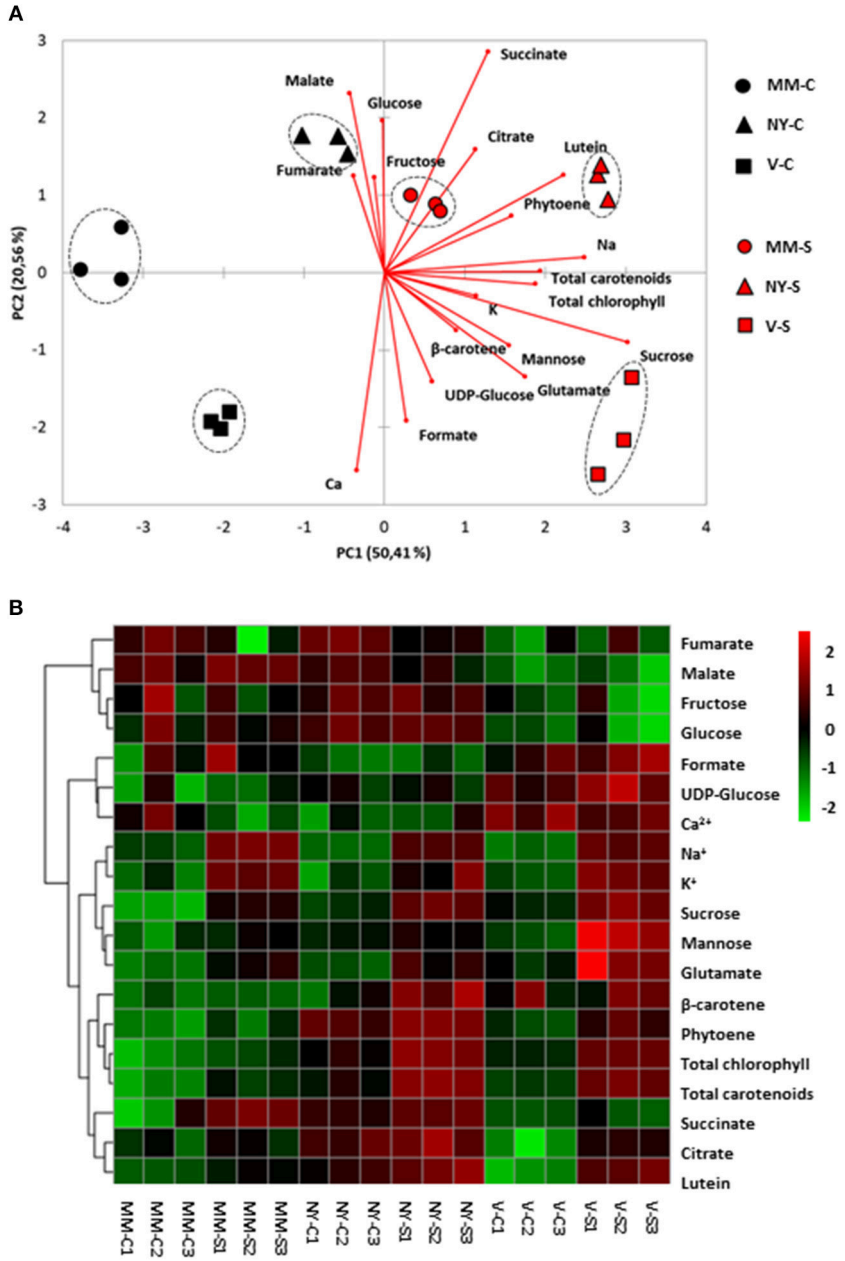

FIGURE 5 | Relative metabolites and cations contents in green fruits of Moneymaker (MM), Negro Yeste (NY), and Verdal (V) from plants grown under control and salt stress(100 mM NaCl during 70 days) conditions. **(A)** Non-supervised principal component analysis (PCA-biplot) and **(B)** heatmap analysis representing of the major sources of variability. Color scale represents the variation in the relative concentration of compounds, from high (red) to low (green) contents.

three tomato varieties in the same organ. In fruits, however, sucrose levels were significantly higher in NY and V landraces compared with MM and, moreover, these levels increased with salt stress (**Table 2**). In this sense salinity tolerance of tomato has been related with higher sucrose transport from source organs to sink organs (Balibrea et al., 2000). But sucrose accumulation in fruits of both landraces may proceed partially because of enhanced exportation from source leaves and partially because of proper biosynthesis within the fruit, since fruit photosynthesis also contributes to sugar accumulation (Lytovchenko et al., 2011). Total chlorophylls were significantly higher in green fruits of NY and V compared with MM in control condition (108 and 63%, respectively), and this content even increased to a higher level under salt stress (134 and 108% in NY and V green fruits, respectively) (**Figure 3**). Taken together, the high levels of sucrose and chlorophylls found in fruits seem to be related with sustainable production under salt stress and with improved fruit quality independently of the environmental conditions.

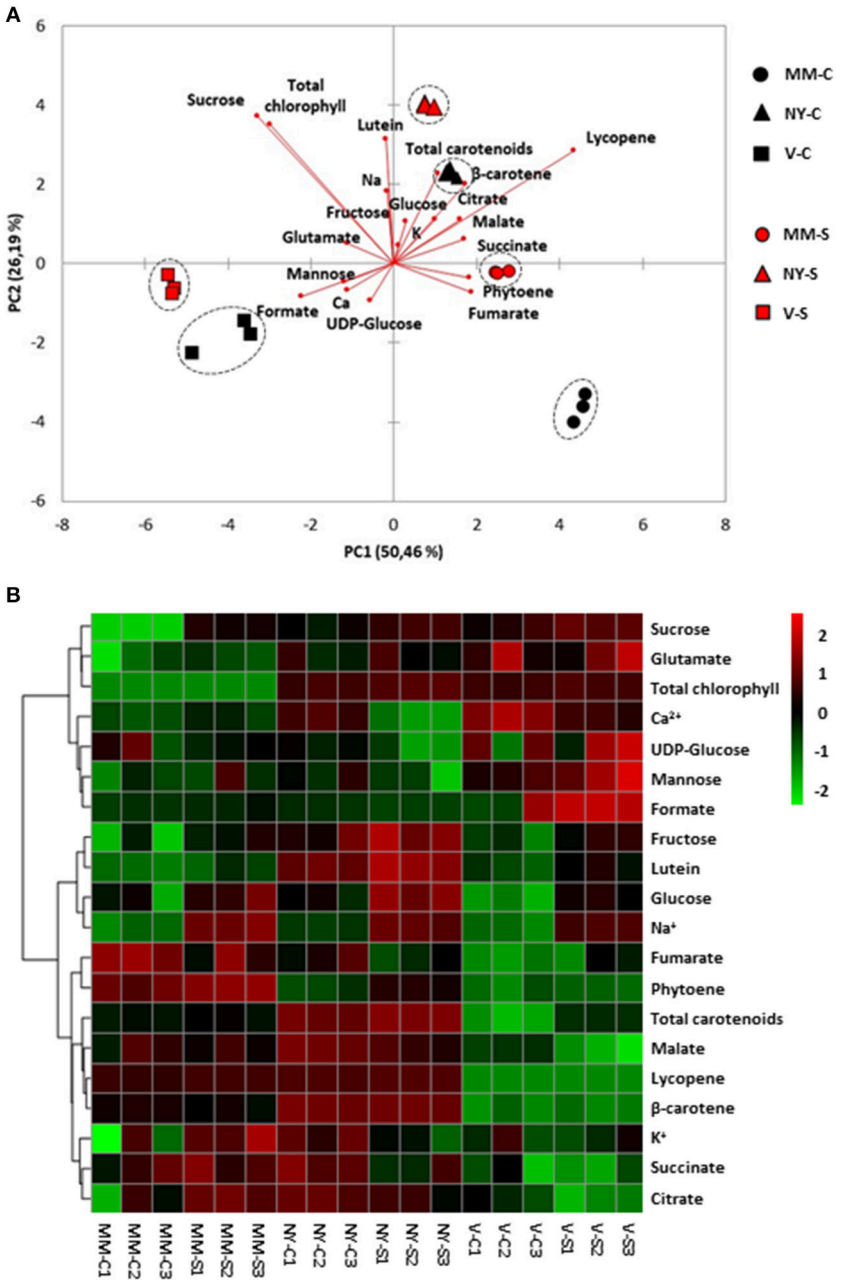

FIGURE 6 | Relative metabolites and cations contents in ripe fruits of Moneymaker (MM), Negro Yeste (NY), and Verdal (V) from plants grown under control and salt stress (100 mM NaCl during 70 days) conditions. **(A)** Non-supervised principal component analysis (PCA-biplot) and **(B)** heatmap analysis representing the major sources of variability. Color scale represents the variation in the relative concentration of compounds, from high (red) to low (green) contents.

The Improved Fruit Quality of Negro Yeste Traditional Tomato Variety Is Associated to High Carotenoids Levels

Changes in metabolites occurring in NY fruits compared with MM ones are represented in biochemical pathways for green and ripe fruits (**Figure 7**). Interestingly, the main changes are already observed in green fruits, as increased levels of sucrose, total chlorophylls, and phytoene, β-carotene and lutein carotenoids are found in control condition as well as in salt-treated plants. In ripe fruits, when lycopene accumulation from phytoene occurs in tomato fruits, the only difference observed with respect to green fruits is the phytoene reduction and lycopene rise in NY compared with MM, which reflects its higher capacity of biosynthesis of lycopene. Moreover, the contents of two carotenoids, β-carotene and lutein, were found at a higher level in fruits from salt-treated plants than from

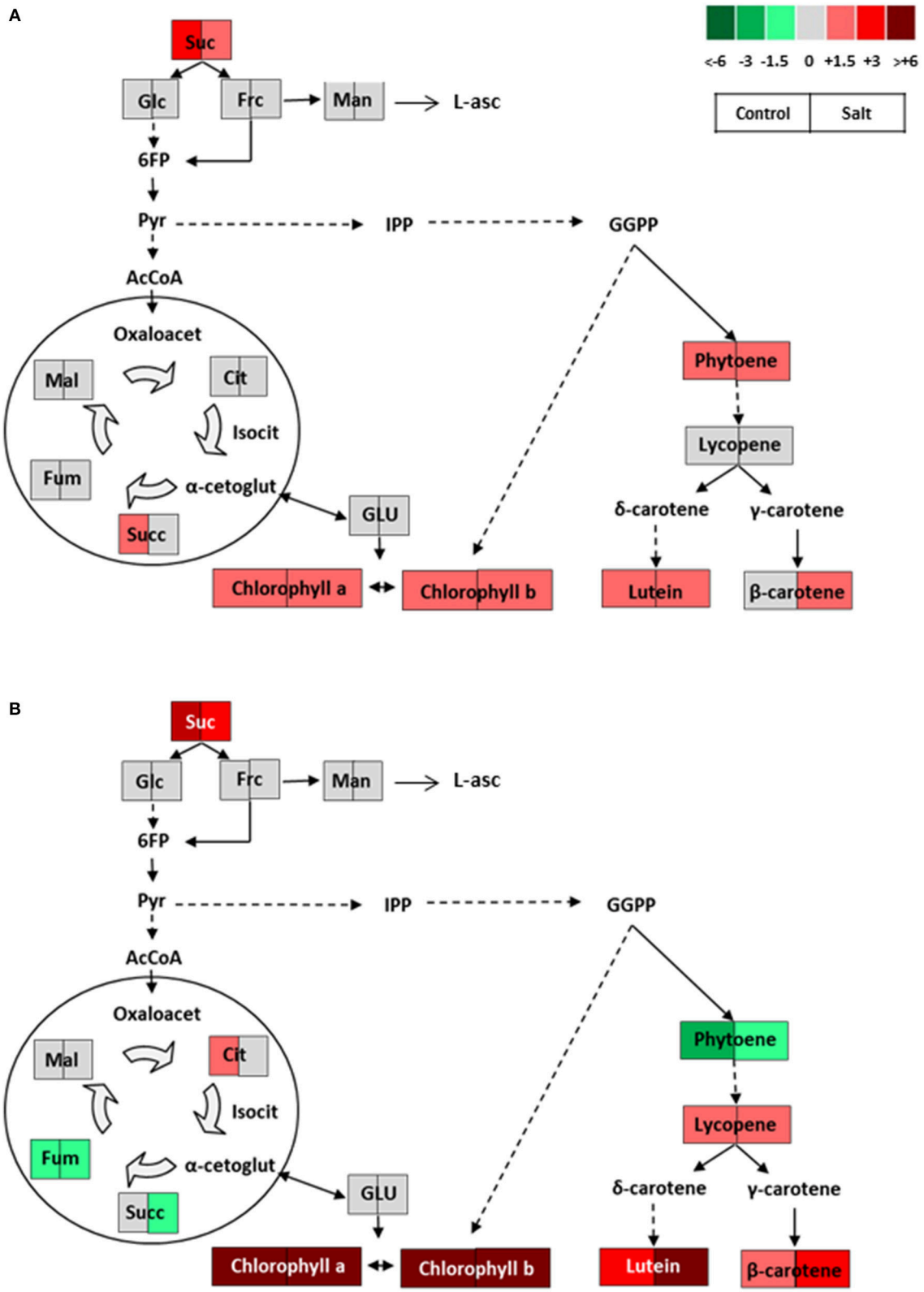

FIGURE 7 | Schematic representations of the metabolic changes occurring in **(A)** green and **(B)** ripe fruits of NegroYeste from plants grown without stress (control) and with salt stress (100 mM NaCl during 70 days). Data were normalized to Moneymaker. Color scale is used to display the different amount of metabolite in terms of fold-change relative to the level in the appropriate control. Suc, sucrose; Glc, glucose; Frc, fructose; Man, mannose; L-asc, L-ascorbic acid; 6FP, fructose-6-phosphate; Pyr, pyruvate; IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate; AcCoA, acetylCoA; Oxaloacet, oxaloacetate; Cit, citrate; Isocit, isocitrate; α-cetoglut, α-cetoglutarate; Succ, succinate; Fum, fumarate; Mal, malate; GLU, glutamate.

control ones. The idea that stress may affect the metabolism of carotenoids in tomato fruit makes sense because many carotenoids are powerful antioxidants and some of them are able to dissipate the excess of absorbed energy caused by the stressful condition in the xanthophyll cycle (Dall'Osto et al., 2013).

It is well-known that carotenoid biosynthesis and chlorophyll degradation pathways are closely related to color development in tomato fruit (Kang et al., 2017), and it is precisely the lack of chlorophyll degradation together with the high accumulation of lycopene what lead to fruits of dark red color in this NY variety. Other important questions to elucidate would be whether the high accumulation of carotenoids in NY fruits is a consequence of their high chlorophylls and sucrose levels. Regarding chlorophylls there are evidences that tomato fruits with more active chloroplasts at the green developmental stage can lead to ripe tomatoes with more active chromoplasts, producing higher amounts of carotenoids (Egea et al., 2010; Liu et al., 2015 and references therein). Moreover, carbohydrates may indirectly influence carotenoids through plastid development in fruits, pointing out that the sheer size of the biosynthetic machinery may be as important as the abundance or the activity of enzymes involved in the biosynthetic pathway of carotenoids (Fanciullino et al., 2014). Taken together the results found regarding carotenoids in relation with other metabolites in NY, this variety may serve as a model to advance in our knowledge of the key processes involved in carotenoid accumulation in tomato fruit. Moreover, it is interesting to point out that the high levels of carotenoids in NY fruits are found independently of the environmental conditions, that is to say, this landrace shows an improved fruit quality without stress and maintains and even increases its quality under a salt stress level relatively high for a sustainable production. In sum, NY could be useful for cultivation in a wide range of stress levels but especially moderate level, where fruit yield reduction would be minimum and, in case of salt stress, the salinity degree would be within a realistic agronomic scenario of arid and semi-arid cultivation areas where moderate saline waters are used in irrigation.

The Metabolism of Ripe Fruits of Verdal Traditional Tomato Variety, Showing a Disrupted Carotenoids Biosynthesis, Is Redirected Toward Formate Accumulation

In green fruits the schematic representation of metabolic changes found in V (**Figure 8**) is quite similar to that observed in NY (**Figure 7**), as levels of sucrose, chlorophylls, and carotenoids are higher in V compared with MM, although in case of the last group of metabolites the changes are limited to salt stress (**Figure 8**). Interestingly, the biggest differences between both landraces appear in the metabolic profiles of ripe fruits since, contrarily to NY, the V variety does not accumulate lycopene, an expected result according to the orange-greenish color of its ripe fruits, and levels of phytoene and β-carotene were reduced with respect to MM, being lutein the only carotenoid maintaining a similar level compared with the commercial cultivar. It is known that, in addition to carotenoids biosynthesis, several metabolic pathways are also derived from geranylgeranyl pyrophosphate (GGPP), like biosynthesis of chlorophylls and other key photosynthesis-related compounds (plastoquinones, phylloquinones, and tocopherols), as well as hormones (gibberellins, ABA, and strigolactones), and finally monoterpenes (Liu et al., 2015). In V fruits the disruption of carotenoids biosynthesis observed at ripe stage might induce the redirection of the metabolic flux toward other pathways. In addition to the high levels of chlorophylls, V fruits showed increased levels of glutamate and mannose in ripe fruits from plants grown in control and salt stress conditions (**Figure 8**). The glutamate rise observed in V fruits is interesting from a fruit quality point of view, since glutamate is a high-valued nutrient (Shinozaki and Ezura, 2016). Its high content could also be related to the higher accumulation of photosynthetic pigments, as this amino acid is the first precursor for the tetrapyrrole ring biosynthesis and an increase in its production may lead to an elevated flux toward chlorophyll accumulation (von Wettstein et al., 1995). Taking into account that the mannose metabolic pathway contributes to increase the ascorbate level in ripe tomato fruits (Badejo et al., 2012), the increased levels of mannose in V fruits might be related with biosynthesis of vitamin C (L-ascorbic acid).

In the "non-cyclic" partial TCA cycle, one branch produces citrate which can be transformed in isocitrate, 2-oxoglutarate or their derivatives (including glutamate), while the other branch produces malate or fumarate and even succinate (Igamberdiev and Eprintsev, 2016). According to the reduced levels of succinate, fumarate, and malate found in V fruits, the second branch was clearly reduced in fruits from this landrace, which could be associated with the increased sugars and total soluble solids observed in them (Centeno et al., 2011). However, V fruits could divert the metabolic flux to the other branch of the TCA, the one derived from isocitrate to render oxalate (**Figure 8**). One of the major pathways for efficient catabolism of oxalate is via its decarboxylation, as decarboxylases catabolize oxalate directly to formate, and CO_2 (Chakraborty et al., 2013). We have observed remarkably high levels of formate in V ripe fruits and, moreover, this increase was higher in fruits from plants subjected to salt stress**.** Furthermore, the increased levels of formate and Ca^{2+} found in V fruits may be related, as Ca^{2+} is sequestered by oxalic acid, and if this organic acid is being catabolized at a higher degree, free Ca^{2+} is transported to the cytoplasm from the vacuole by tonoplast antiporters (Chakraborty et al., 2013). It is interesting to point out that most analytical studies about distribution of oxalates in plants have been focused on their possible Ca^{2+}-sequestering activity and its influence in the human diet, as in its ionic form it performs critical functions in metabolism. Also Ca^{2+} deficiency is the most common nutritional problem affecting tomato fruit development (Nakata, 2003; Park et al., 2005). Finally, taking into account that tomato fruits accumulate considerable amounts of oxalate, a common anti-nutrient in the human diet, formate accumulation in ripe V fruits may be a very interesting trait in fruit quality breeding programs

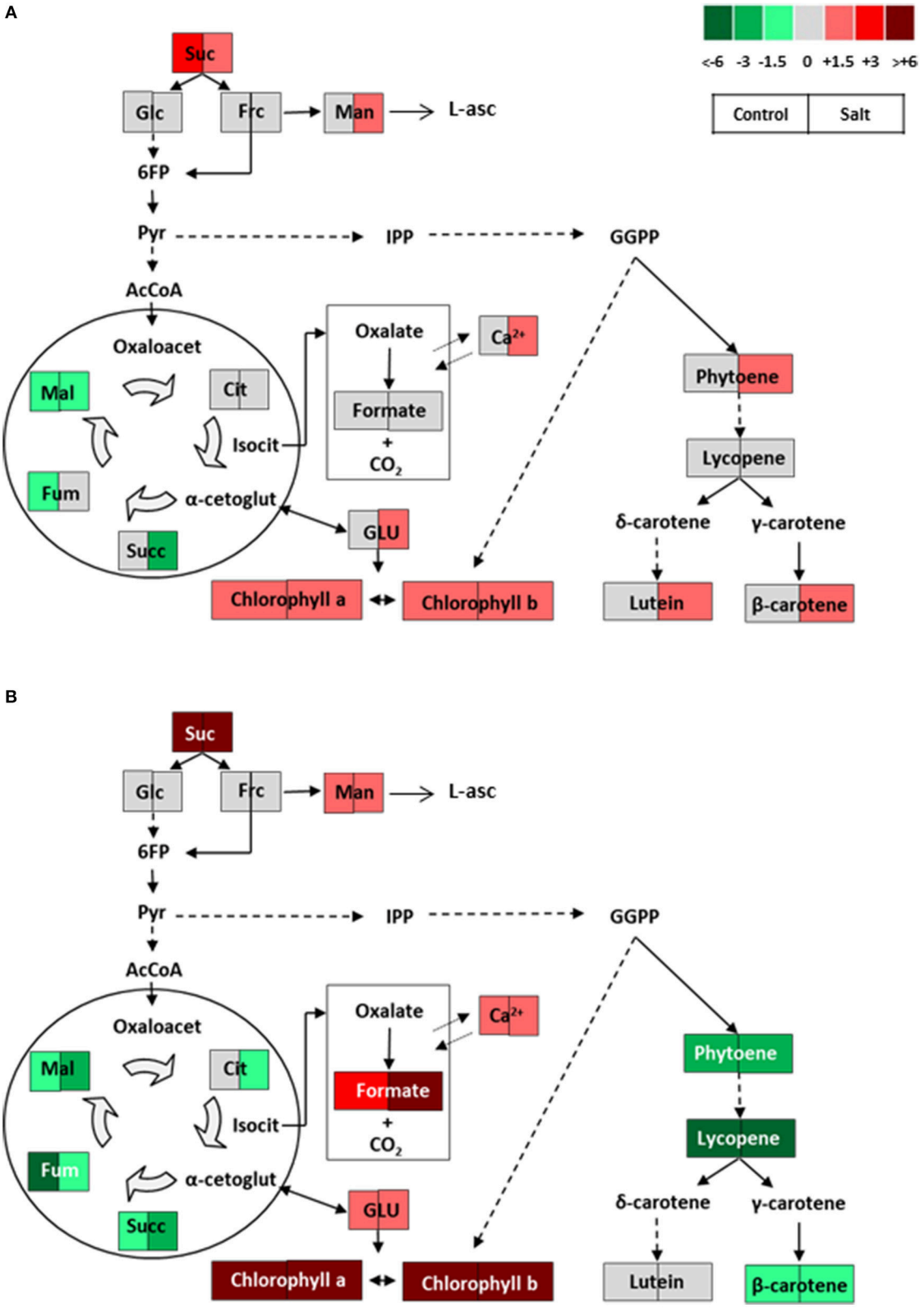

FIGURE 8 | Schematic representations of the metabolic changes occurring **(A)** in green and **(B)** in ripe fruits of Verdal from plants grown without stress (control) and with salt stress (100 mM NaCl during 70 days). Data were normalized to Moneymaker. Color scale is used to display the different amount of metabolite in terms of fold-change relative to the level in the appropriate control. Suc, sucrose; Glc, glucose; Frc, fructose; Man, mannose; L-asc, L-ascorbic acid; 6FP, fructose-6-phosphate; Pyr, pyruvate; IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate; AcCoA, acetylCoA; Oxaloacet, oxaloacetate; Cit, citrate; Isocit, isocitrate; α-cetoglut, α-cetoglutarate; Succ, succinate; Fum, fumarate; Mal, malate; GLU, glutamate.

targeting tomato. Hitherto, the priorities in such breeding programs have been focused in rising primary and secondary metabolites, especially carotenoids, but in much less extension they have dealt with reduction of oxalic acid (Chakraborty et al., 2013).

In conclusion, both landraces show contrasting metabolic patterns to improve fruit quality, one (NY) increasing carotenoids and the other (V) redirecting the metabolic pathway toward other metabolites still unknown. Therefore, these varieties may be very interesting resources to be used in a near future to obtain new lines with improved fruit quality and enhanced fruit yield when grown under adverse environmental conditions.

AUTHOR CONTRIBUTIONS

IM performed the experiments and data analysis, and contributed to the manuscript preparation. IA and FP helped in analysis of leaf and fruit materials. EP and FF critically revised the manuscript for important intellectual content. JE-F collected and selected the landraces. IE and MB conceived and designed the research and wrote the manuscript. All authors read and approved the manuscript.

FUNDING

This work was supported by grant Ref. 19305/PI/14 from the SENECA Foundation (science and technology agency of the Region of Murcia, Spain). IM thanks the FAPESP for her postdoctoral fellowship Ref. 2017/12311-1 and financial support Ref. 2013/07914-8.

ACKNOWLEDGMENTS

We thank the metabolomics core facility of CEBAS for their support on the metabolomics analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01778/full#supplementary-material

REFERENCES

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts polyphenoloxidase in *beta vulgaris*. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1

Badejo, A. A., Wada, K., Gao, Y., Maruta, T., Sawa, Y., Shigeoka, S., et al. (2012). Translocation and the alternative D-galacturonate pathway contribute to increasing the ascorbate level in ripening tomato fruits together with the D-mannose/L-galactose pathway. *J. Exp. Bot.* 63, 229–239. doi: 10.1093/jxb/err275

Balibrea, M. E., Dell'Amico, J., Bolarín, M. C., and Pérez-Alfocea, F. (2000). Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. *Physiol. Plant.*110, 503–511. doi: 10.1111/j.1399-3054.2000.1100412.x

Bergougnoux, V. (2014). The history of tomato: from domestication to biopharming. *Biotechnol. Adv.* 32, 170–189. doi: 10.1016/j.biotechadv.2013.11.003

Campos, J. F., Cara, B., Pérez-Martín, F., Pineda, B., Egea, I., Flores, F. B., et al. (2016). The tomato mutant *ars1* (*altered response to salt stress 1*) identifies an R1-typeMYB transcription factor involved in stomatal closure under salt acclimation. *Plant Biotechnol. J.* 14, 1345–1356. doi: 10.1111/pbi.12498

Centeno, D. C., Osorio, S., Nunes-Nesi, A., Bertolo, A. L. F., Carneiro, R. T., Araujo, W. L., et al. (2011). Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *Plant Cell* 23, 162–184. doi: 10.1105/tpc.109.072231

Chakraborty, N., Ghosh, R., Ghosh, S., Narula, K., Tayal, R., Datta, A., et al. (2013). Reduction of oxalate levels in tomato fruit and consequent metabolic remodeling following overexpression of a fungal oxalate decarboxylase. *Plant Physiol.* 162, 364–378. doi: 10.1104/pp.112.209197

Choi, H. K., Choi, Y. H., Verberne, M., Lefeber, A. W., Erkelens, C., and Verpoorte, R. (2004). Metabolic fingerprinting of wild type and transgenic tobacco plants by 1H NMR and multivariate analysis technique. *Phytochemistry* 65, 857–864. doi: 10.1016/j.phytochem.2004.01.019

Choi, Y. H., Kim, H. K., Linthorst, H. J. M., Hollander, J. G., Lefeber, A. W. M., Erkelens, C., et al. (2006). NMR metabolomics to revisit the tobacco mosaic virus infection in *Nicotiana tabacum* leaves. *J. Nat. Prod.* 69, 742–748. doi: 10.1021/np050535b

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4,0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* 46, W486–W494. doi: 10.1093/nar/gky310

Cocaliadis, M. F., Fernandez-Munoz, R., Pons, C., Orzaez, D., and Granell, A. (2014). Increasing tomato fruit quality by enhancing fruit chloroplast function. A double-edged sword? *J. Exp. Bot.* 65, 4589–4598. doi: 10.1093/jxb/eru165

Colla, G., Rouphael, Y., Cardarelli, M., Svecova, E., Rea, E., and Lucini, L. (2013). Effects of saline stress on mineral composition, phenolics acids and flavonoids in leaves ofartichoke and cardoon genotypes grown in floating system. *J. Sci. Food Agric.* 93, 1119–1127. doi: 10.1002/jsfa.5861

Cuartero, J., Bolarín, M. C., Asíns, M. J., and Moreno, V. (2006). Increasing salt tolerance in the tomato. *J. Exp. Bot.* 57, 1045–1058. doi: 10.1093/jxb/erj102

Dall'Osto, L., Piques, M., Ronzani, M., Molesini, B., Alboresi, A., Cazzaniga, S., et al. (2013). The Arabidopsis *nox* mutant lacking carotene hydroxylase activity reveals a critical role for xanthophylls in photosystem I biogenesis. *Plant Cell* 25, 591–608. doi: 10.1105/tpc.112.108621

D'Esposito, D., Ferriello, F., Dal Molin, A., Diretto, G., Sacco, A., Minio, A., et al. (2017). Unraveling the complexity of transcriptomic, metabolomic and quality environmental response of tomato fruit. *BMC Plant Biol.* 17:66. doi: 10.1186/s12870-017-1008-4

Dwivedi, S. L., van Bueren, E. T. L., Ceccarelli, S., Grando, S., Upadhyaya, H. D., and Ortiz, R. (2017). Diversifying food systems in the pursuit of sustainable food production and healthy diets. *Trends Plant Sci.* 22, 843–856. doi: 10.1016/j.tplants.2017.06.011

Egea, I., Albaladejo, I., Morales, B., Egea-Fernández, J. M., Flores, F. B., and Bolarín, M. C. (2017). Estrategias para mejorar la calidad nutricional del tomate en condiciones mediterráneas. *Agricola Vergel* 401, 171–173.

Egea, I., Barsan, C., Bian, W., Purgatto, E., Latche, A., Chervin, C., et al. (2010). Chromoplast differentiation: current status and perspectives. *Plant Cell Physiol.* 51, 1601–1611. doi: 10.1093/pcp/pcq136

Egea, I., Pineda, B., Ortíz-Atienza, A., Plasencia, F. A., Drevensek, S., García-Sogo, B., et al. (2018). The SlCBL10 calcineurin B-Like protein ensures plant growth under salt stress by regulating Na^+ and Ca^{2+}homeostasis. *Plant Physiol.* 176, 1676–1693. doi: 10.1104/pp.17.01605

Fanciullino, A. L., Bidel, L. P. R., and Urban, L. (2014). Carotenoid responses to environmental stimuli: integrating redox and carbon controls into a fruit model. *Plant Cell Environ.* 37, 273–289. doi: 10.1111/pce.12153

FAOSTAT (2016). *UN Food and Agriculture Organization statistics [Online].* Available online at http://www.fao.org/faostat

Fraser, P. D., and Bramley, P. M. (2004). The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* 43, 228–265. doi: 10.1016/j.plipres.2003.10.002

Frison, E. A., Cherfas, J., and Hodgkin, T. (2011). Agricultural biodiversity is essential for a sustainable improvement in food and nutrition security. *Sustainability* 3, 238–253. doi: 10.3390/su3010238

García-Abellan, J. O., Egea, I., Pineda, B., Sanchez-Bel, P., Belver, A., Garcia-Sogo, B., et al. (2014). Heterologous expression of the yeast *HAL5* gene in tomato enhances salt tolerance by reducing shoot Na^+ accumulation in the long term. *Physiol. Plant.*152, 700–713. doi: 10.1111/ppl.12217

Gascuel, Q., Diretto, G., Monforte, A. J., Fortes, A. M., and Granell, A. (2017). Use of natural diversity and biotechnology to increase the quality and nutritional content of tomato and grape. *Front. Plant Sci.* 8:652. doi: 10.3389/fpls.2017.00652

Gilliham, M., Dayod, M., Hocking, B. J., Xu, B., Conn, S. J., Kaiser, B. N., et al. (2011). Calcium delivery and storage in plant leaves: exploring the link with water flow. *J. Exp. Bot.* 62, 2233–2250. doi: 10.1093/jxb/err111

Hoagland, D. R., and Arnon, D. I. (1950). *The Water-Culture Method for Growing Plants Without Soil, 2nd Edn*. Berkeley, CA: The college of Agriculture, University of California.

Hunter, R. S. (1975). *The Measurement of Appearance*. New York, NY: John Wiley & Sons Publisher.

Igamberdiev, A. U., and Eprintsev, A. T. (2016). Organic acids: the pools of fixed carbon involved in redox regulation and energy balance in higher plants. *Front. Plant Sci.* 7:1042. doi: 10.3389/fpls.2016.01042

Kang, S.-I., Hwang, I., Goswani, G., Jung, H. J., Nath, U. K., Yoo, H.-J., et al. (2017). Molecular insights reveal *Psy1*, *SGR*, and *SlMYB12* genes are associated with diverse fruit color pigments in tomato (*Solanum lycopersicum* L.). *Molecules* 22:2180. doi: 10.3390/molecules22122180

Kyriacou, M. C., and Rouphael, Y. (2018). Towards a new definition of quality for fresh fruits and vegetables. *Sci. Hortic.* 234, 463–469. doi: 10.1016/j.scienta.2017.09.046

Liu, L., Shao, Z., Zhang, M., and Wang, Q. (2015). Regulation of carotenoid metabolism in tomato. *Mol. Plant* 8, 28–39. doi: 10.1016/j.molp.2014.11.006

Lytovchenko, A., Eickmeier, I., Pons, C., Osorio, S., Szecowka, M., Lehmberg, K., et al. (2011). Tomato fruit photosynthesis is seemingly unimportant in primary metabolism and ripening but plays a considerable role in seed development. *Plant Physiol.* 157, 1650–1663. doi: 10.1104/pp.111.186874

McQuinn, R. P., Wong, B., and Giovannoni, J. J. (2018). *AtPDS* overexpression in tomato: exposing unique patterns of carotenoid self-regulation and an alternative strategy for the enhancement of fruit carotenoid content. *Plant Biotechnol. J.* 16, 482–494. doi: 10.1111/pbi.12789

Nagata, M., and Yamashita, I. (1992). Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. *J. Jpn. Soc. Food Sci. Technol.* 39, 925–928. doi: 10.3136/nskkk1962.39.925

Nakata, P. A. (2003). Advances in our understanding of calcium oxalate crystal formation and function in plants. *Plant Sci.* 164, 901–909. doi: 10.1016/S0168-9452(03)00120-1

Osorio, S., Ruan, Y. L., and Fernie, A. (2014). An update on source-to-sink carbon partitioning in tomato.*Front. Plant Sci.* 5:516. doi: 10.3389/fpls.2014.00516

Park, S., Cheng, N. H., Pittman, J. K., Yoo, K. S., Park, J., Smith, R. H., et al. (2005). Increased calcium levels and prolonged shelf life in tomatoes expressing Arabidopsis H^+/Ca^{2+} transporters. *Plant Physiol.* 139, 1194–1206. doi: 10.1104/pp.105.066266

Ringeisen, B., Barrett, D. M., and Stroeve, P. (2014). Concentrated solar drying of tomatoes. *Energy Sustain. Dev*. 19, 47–55. doi: 10.1016/j.esd.2013.11.006

Rivera, S., and Canela, R. (2012). Influence of sample processing on the analysis of carotenoids in maize. *Molecules* 17, 11255–11268. doi: 10.3390/molecules170911255

Rivera, S. M., Vilaró, F., Zhu, C., Bai, C., Farré, G., Christou, P., et al. (2013). Fast quantitative method for the analysis of carotenoids in transgenic maize. *J. Agric. Food Chem.* 61, 5279–5285. doi: 10.1021/jf400694z

Rouphael, Y., Cardarelli, M., Bassal, A., Leonardi, C., Giuffrida, F., and Colla, G. (2012). Vegetable quality as affected by genetic, agronomic and environmental factors. *J. Food Agric. Environ.*10, 680–688. doi: 10.1234/4.2012.3485

Schreiner, M., Korn, M., Stenger, M., Holzgreve, L., andAltmann, M. (2013). Current understandingand use of quality characteristics of horticulture products. *Sci. Hortic.* 163, 63–69. doi: 10.1016/j.scienta.2013.09.027

Schwarz, D., Thompson, A. J., and Kläring, H. P. (2014). Guidelines to use tomato in experiments with a controlled environment. *Front. Plant Sci*. 5:625. doi: 10.3389/fpls.2014.00625

Sérino, S., Gomez, L., Costagliola, G., and Gautier, H. (2009). HPLC assay of tomato carotenoids: validation of a rapid microextraction technique. *J. Agric. Food Chem*. 57, 8753–8760. doi: 10.1021/jf902113n

Sharoni, Y., Linnewiel-Hermoni, K., Khanin, M., Salman, H., Veprik, A., Danilenko, M., et al. (2012). Carotenoids and apocarotenoids in cellular signaling related to cancer: a review. *Mol. Nutr. Food Res.* 56, 259–269. doi: 10.1002/mnfr.201100311

Shinozaki, Y., and Ezura, K. (2016). "Tomato fruit set and its modification using molecular breeding techniques," in *Functional Genomics and Biotechnology in Solanaceae and Cucurbitaceae Crops. Biotechnology in Agriculture and Forestry* Vol. 70, eds H. Ezura, T. Ariizumi, J. Garcia-Mas, and J. Rose Berlin (Heidelberg: Springer-Verlag), 93–112.

Sonnewald, U., and Fernie, A. R. (2018). Next-generation strategies for understanding and influencing source-sink relations in crop plants. *Curr. Opin. Plant Biol.* 43, 63–70. doi: 10.1016/j.pbi.2018.01.004

von Wettstein, D., Gough, S., and Kannangara, C. G. (1995). Chorophyll biosynthesis. *Plant Cell* 7, 1039–1057. doi: 10.1105/tpc.7.7.1039

Wu, M., and Kubota, C. (2008). Effects of high electrical conductivity of nutrient solution and its application timing on lycopene, chlorophyll and sugar concentrations of hydroponic tomatoes during ripening. *Sci. Hortic.*116, 122–129. doi: 10.1016/j.scienta.2007.11.014

Zhu, G., Wang, S., Huang, Z., Zhang, S., Liao, Q., Zhang, C., et al. (2018). Rewiring of the fruit metabolome in tomato breeding. *Cell* 172, 249–261. doi: 10.1016/j.cell.2017.12.019

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 December 2018
doi: 10.3389/fpls.2018.01774

Insights Into the Adaptation to Greenhouse Cultivation of the Traditional Mediterranean Long Shelf-Life Tomato Carrying the *alc* Mutation: A Multi-Trait Comparison of Landraces, Selections, and Hybrids in Open Field and Greenhouse

Maria R. Figàs[1], Jaime Prohens[1], María D. Raigón[1], Leandro Pereira-Dias[1], Cristina Casanova[1], María D. García-Martínez[1], Elena Rosa[1], Elena Soler[1], Mariola Plazas[2] and Salvador Soler[1]*

[1] *Institut de Conservació i Millora de l'Agrodiversitat Valenciana, Universitat Politècnica de València, Valencia, Spain,* [2] *Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universitat Politècnica de València, Valencia, Spain*

OPEN ACCESS

Edited by:
Isabel C. F. R. Ferreira, Polytechnic Institute of Bragança, Portugal

Reviewed by:
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy
Francesco Di Gioia, Pennsylvania State University, United States

***Correspondence:**
Jaime Prohens
jprohens@btc.upv.es

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *31 May 2018*
Accepted: *15 November 2018*
Published: *04 December 2018*

Citation:
Figàs MR, Prohens J, Raigón MD, Pereira-Dias L, Casanova C, García-Martínez MD, Rosa E, Soler E, Plazas M and Soler S (2018) Insights Into the Adaptation to Greenhouse Cultivation of the Traditional Mediterranean Long Shelf-Life Tomato Carrying the alc Mutation: A Multi-Trait Comparison of Landraces, Selections, and Hybrids in Open Field and Greenhouse. Front. Plant Sci. 9:1774. doi: 10.3389/fpls.2018.01774

Long shelf-life tomato (*Solanum lycopersicum*) landraces, characterized by carrying the *alc* allele in the NOR.NAC locus, have been traditionally cultivated in the Mediterranean region. These materials are adapted to open field conditions under low input conditions. However, cultivation under greenhouse is expanding fueled by increasing demand of these traditional tomatoes. We hypothesize that the large diversity in the long shelf-life landraces and derived materials can be exploited for adaptation to these new cultivation conditions. We have evaluated 12 varieties (seven landraces, three selections and two hybrids) carrying the *alc* mutation under open field (OF) and greenhouse (GH) cultivation, and evaluated them for 52 morphological, agronomic, chemical properties, and chemical composition descriptors. All descriptors, except six morphological ones, were variable. The variety effect was the greatest contributor to variation for most morphological traits, as well as for fruit weight, fruit shape, dry matter, and soluble solids content. However, significant environmental and genotype × environment interaction were found for 36 and 42 descriptors, respectively. Fruits from GH plants had lower weight and firmness and were less red than those from OF. On average, in GH yield was 35% lower and daily fruit weight loss in post-harvest 41% higher than in OF. However, fruits from GH had on average higher dry matter and soluble solids contents, antioxidant activity, glucose, fructose, and ascorbic acid concentrations, but lower contents in lycopene and β-carotene than those from OF. A principal components analysis clearly separated varieties according to the cultivation environment. However, the distribution pattern of varieties within each of the two clusters (GH and OF) was similar, despite the strong G × E

interaction for many descriptors. Landraces from the same origin plotted in the same area of each cluster, and selections and hybrids plotted together with the landraces. The results reveal a high impact of the cultivation environment on morphological, agronomic, chemical properties, and chemical composition of Mediterranean long shelf-life traditional tomato varieties. This suggests that breeding programs specifically focused to adaptation to greenhouse conditions should be developed.

Keywords: breeding, cultivation conditions, fruit quality, genotype × environment interaction, selection, *Solanum lycopersicum*, yield

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) landraces with extremely extended long shelf-life, of several months at room temperature, have been traditionally cultivated in Mediterranean regions (Casals et al., 2012; Bota et al., 2014; Mercati et al., 2015). These landraces are commonly known as "de colgar" in Spanish, "de penjar" or "de ramellet" in Catalan, or "da serbo" in Italian (Bota et al., 2014; Cortés-Olmos et al., 2015; Mercati et al., 2015). These local names make reference to its conservation by hanging in strings ("de colgar" and "de penjar"), to the fact that they normally set in clusters ("de ramellet"), or that have a long storage period ("da serbo"). Before the generalized advent of refrigerators and greenhouse cultivation Mediterranean long shelf-life tomatoes, when stored in ventilated rooms typically hanging in strings with the fruits threaded through the pedicel, allowed the availability of fresh tomatoes throughout the winter time (Casals et al., 2012; Bota et al., 2014; Mercati et al., 2015). This characteristic made its cultivation very popular in several Mediterranean areas, like in the island of Majorca in the first half of the twentieth century (Fairchild, 1927). Despite the general loss of prominence of the Mediterranean long shelf-life tomatoes during the second half of the twentieth century, in the last years there has been an increased interest in these local varieties for their utilization in the traditional local gastronomy (Romero del Castillo et al., 2014). These varieties also are of interest for their resilience and drought tolerance as adaptive traits against climatic change (Maamar et al., 2015; Fullana-Pericàs et al., 2017, 2018).

Several studies reveal that the extended shelf-life of most of the Mediterranean long shelf-life tomatoes of the Spanish "de colgar," "de penjar," and "de ramellet" typologies is caused by the *alc* (*alcobaça*) allele of the NAC. NOR gene (Casals et al., 2012; Bota et al., 2014). The *alc* allele also accounts for the long shelf-life of the Italian "da serbo" type (Mercati et al., 2015), but not for other Italian long shelf-life varieties like Corbarino and Lucariello (Tranchida-Lombardo et al., 2018). The *alc* mutation confers a specific phenotype associated to a delayed ripening and reduced lycopene/β-carotene ratio in the fruits (Mutschler et al., 1992; Figàs et al., 2015b), and is found in many different genetic backgrounds (Cebolla-Cornejo et al., 2013). This indicates that throughout the years traditional farmers made an efficient selection of a diverse set of tomato landraces carrying this mutation. As a result, there are many local varieties in the Mediterranean region with the *alc* mutation, with a wide morphological diversity (Cebolla-Cornejo et al., 2013; Bota et al., 2014; Figàs et al., 2015a; Mercati et al., 2015). However, because fruit size in these long shelf-life tomatoes is negatively correlated with the post-harvest conservation period (Casals et al., 2012), fruits are generally smaller than those of standard tomatoes (Bota et al., 2014; Figàs et al., 2015a). Remarkably, these Mediterranean long shelf-life tomatoes use to have a higher dry matter content than standard varieties (Figàs et al., 2015b). The high dry matter content may contribute to its extended post-harvest, and renders them as an interesting material for breeding tomatoes with better flavor (Casals et al., 2011).

The traditional cultivation of the long shelf-life local tomato varieties from the Mediterranean region has been done in the open field with no or reduced irrigation (Mercati et al., 2015; Fullana-Pericàs et al., 2018). The limited availability of water reduced yield dramatically, but improved conservation (Conesa et al., 2014), and decreased the cultivation costs to a minimum, so that even with low yields cultivation was profitable. During the last decades the situation changed completely and modern techniques, including irrigation and increased fertilization have been applied to Mediterranean long shelf-life tomatoes in order to improve yields. In addition, due to increased demand (Romero del Castillo et al., 2014), greenhouse tomato producers started to grow the *alc* traditional long shelf-life tomatoes. Greenhouse cultivation, although more expensive than open field cultivation, allows avoiding costs associated to storage of large quantities of fruits in well-ventilated rooms for long periods. It may also reduce the post-harvest losses due to spoilage of a certain percentage of fruits after months of storage (Casals et al., 2012; Conesa et al., 2014), caused by bruising during harvest and post-harvest handling or due to tomato berries breaking off from the pedicel in fruits hanged on strings. However, these tomato long shelf-life varieties were selected for open field cultivation in the summer season under no or reduced irrigation and low-input conditions (Bota et al., 2014; Mercati et al., 2015; Fullana-Pericàs et al., 2017). As tomato greenhouse conditions involve reduced solar irradiation and high levels of irrigation and fertilization (Peet and Welles, 2005), their adaptation to greenhouse conditions is often suboptimal. Although long shelf-life tomato cultivation has traditionally been based on local landraces (Casals et al., 2012; Bota et al., 2014; Mercati et al., 2015), some local seed companies are marketing selections of this type of tomato and in some cases are producing hybrids with long shelf-life characteristics resulting from the presence of the *alc* mutation (Marín, 2015).

The genetic variation of Mediterranean long shelf-life tomatoes is large (Cebolla-Cornejo et al., 2013; Mercati et al., 2015). Therefore, there are ample opportunities for exploitation of the genotype × environment (G × E) interaction for improving the production and quality of long shelf-life tomatoes under greenhouse. In particular, environmental effects are important for fruit quality, defined by Kyriacou and Rouphael (2018) as "*a dynamic composite of physicochemical properties and evolving consumer perception,*" which embraces organoleptic, nutritional, and bioactive compounds (Hounsome et al., 2008; Barrett et al., 2010; Kaushik et al., 2015). In other works in tomato, G × E interaction in tomato varieties when comparing open field and greenhouse conditions has been very important for both yield and quality traits (Kuti and Konuru, 2005; Roselló et al., 2010; Adalid et al., 2012; Figàs et al., 2018). However, to our knowledge there are no comprehensive evaluations of traits of interest to producers (plant and fruit morphology, agronomic traits), traders (fruit characteristics, post-harvest performance), and consumers (fruit morphology, composition) of a significant number of long shelf-life tomato varieties from different origins and types, including landraces and commercial selections and hybrids.

The aim of the present work is evaluating if the large diversity found among Mediterranean long shelf-life tomatoes carrying the *alc* allele (Cebolla-Cornejo et al., 2013; Bota et al., 2014; Mercati et al., 2015) can be exploited for selecting materials with good adaptation to greenhouse conditions. To test our hypothesis, in this work we evaluate 12 long shelf-life tomato varieties carrying the *alc* mutation from different origins and types (landraces, commercial selections, commercial hybrids) in open field and in greenhouse and characterize them for 52 morphological, agronomic, chemical properties, and chemical composition descriptors. The results obtained will provide relevant information for the enhancement of this varietal type and its adaptation to greenhouse cultivation.

MATERIALS AND METHODS

Plant Materials and Cultivation Conditions

Twelve long shelf-life varieties carrying the *alc* allele were used for the present study (**Figure 1**). Varieties used include: (a) three landraces used for the production of the Valencian Community Quality Mark "Tomata de Penjar" in the Alcalà de Xivert municipality (province of Castellò, mainland Spain) and locally known as "Estrella," "Moradeta," and "Punteta"; (b) the type landrace (UIB-2-70) of the conservation variety "Tomátiga de Ramellet" from Majorca Island (Spain); (c) three landraces from the germplasm bank of Universitat de les Illes Balears collected in Majorca Island (BGIB-018, BGIB-107, BGIB-198), corresponding to the "Tomàtiga de Ramellet" highly variable landrace (Bota et al., 2014); (d) a selection of long shelf-life (*alc*) tomato used for greenhouse cultivation in the Almería province (Spain) called "SEL1"; (e) two commercial varieties corresponding to selections of the long shelf-life (*alc*) tomato type ("Domingo" and "Mallorquín") from Semillas Batlle (Molins de Rei, Barcelona, Spain); and (f) two commercial long shelf-life hybrids ("Palamós F1" and "Manacor F1") both of which are resistant to *Tomato mosaic virus* (ToMV) and to *Tomato spotted wilt virus* (TSWV), and also to *Fusarium oxysporum* f. sp. *lycopersici* in the case of "Manacor F1," from Semillas Fitó (Barcelona, Spain).

The 12 varieties were grown under both open field (OF) and greenhouse (GH), with 10 plants per variety under each of the conditions. Plants in each condition were distributed in a completely randomized design, making a total of 10 replicates with one plant per replicate. Prior to germination, seeds were disinfected with a 1:10 w/v solution of dodecahydrate trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) for 3 h and rinsed three times with distilled water; after that a new round of disinfection was performed with a solution of 0.37% sodium hypochlorite (NaOCl) for 1 h followed by three rinsings of 10 min with distilled water. After that, seeds were left to dry on filter paper for several days under room conditions and then placed in hermetic flasks with dry silica gel for several weeks. After that, seeds were thermotreated at 80°C for 24 h. Disinfected seeds were germinated in commercial substrate seedling trays and transplanted when plantlets had a height of around 12–15 cm. Transplanting of OF and GH trials was performed on 29 April 2016 and 19 February 2016, respectively, and lasted until 27 July 2016 and 25 May 2016. These are typical growing cycles in open field and greenhouse cultivation in the area, and dates used for the transplant are within the usual ranges used by commercial farmers. Minimum, maximum, and average temperatures throughout the cultivation period in OF were, respectively, of 9.3, 31.9, and 22.4°C, while in GH were of 4.9, 32.3, and 18.3°C, respectively. The time course of minimum, maximum and average temperatures throughout the cultivation period is presented in **Figure 2**. The soils of both environments were of the USDA clay-loam soil texture class, with an organic matter of content of 2.72% in OF and 2.64% in GH and a pH of 7.92 in OF and 7.99 in GH.

The open field plot was located in Alcalà de Xivert (Castelló, Spain) in the area of traditional cultivation of the Quality Mark "Tomata de Penjar." Plants were spaced 0.70 m among rows and 0.50 m within rows. The traditional cultivation techniques were performed, and plants were staked with canes and left unpruned. Irrigation was provided through a drip irrigation system depending on the needs of the plant for a total volume of 1356.7 mm, to which 28.4 mm of pluviometry have to be added, making a total of 484.8 l/plant (**Figure 2**). After an initial watering of 6.5 l/plant just after the transplant, 4 l/plant were applied daily until the day 29 after transplant, followed by 5 l/plant per day until the day 53 after transplant, and finally 7 l/plant per day every 2 days until the end of the experiment (day 87 after transplant). Pluviometry was mostly concentrated on days 11 (14.4 mm), 12 (5.6 mm), 20 (3.6 mm), and 24 (2.8 mm), while the rest was scattered in seven different non-consecutive days with a range between 0.2 and 0.4 mm per day. A background fertilization consisting of 2.85 kg/m^2 of poultry manure (2.4% N, 1.0% P, and 1.2% K) was applied before transplant. A top-dressing fertilization of 0.042 kg/m^2 of fertilizer containing 19% N, 6% P_2O_5, and 6% K_2O was

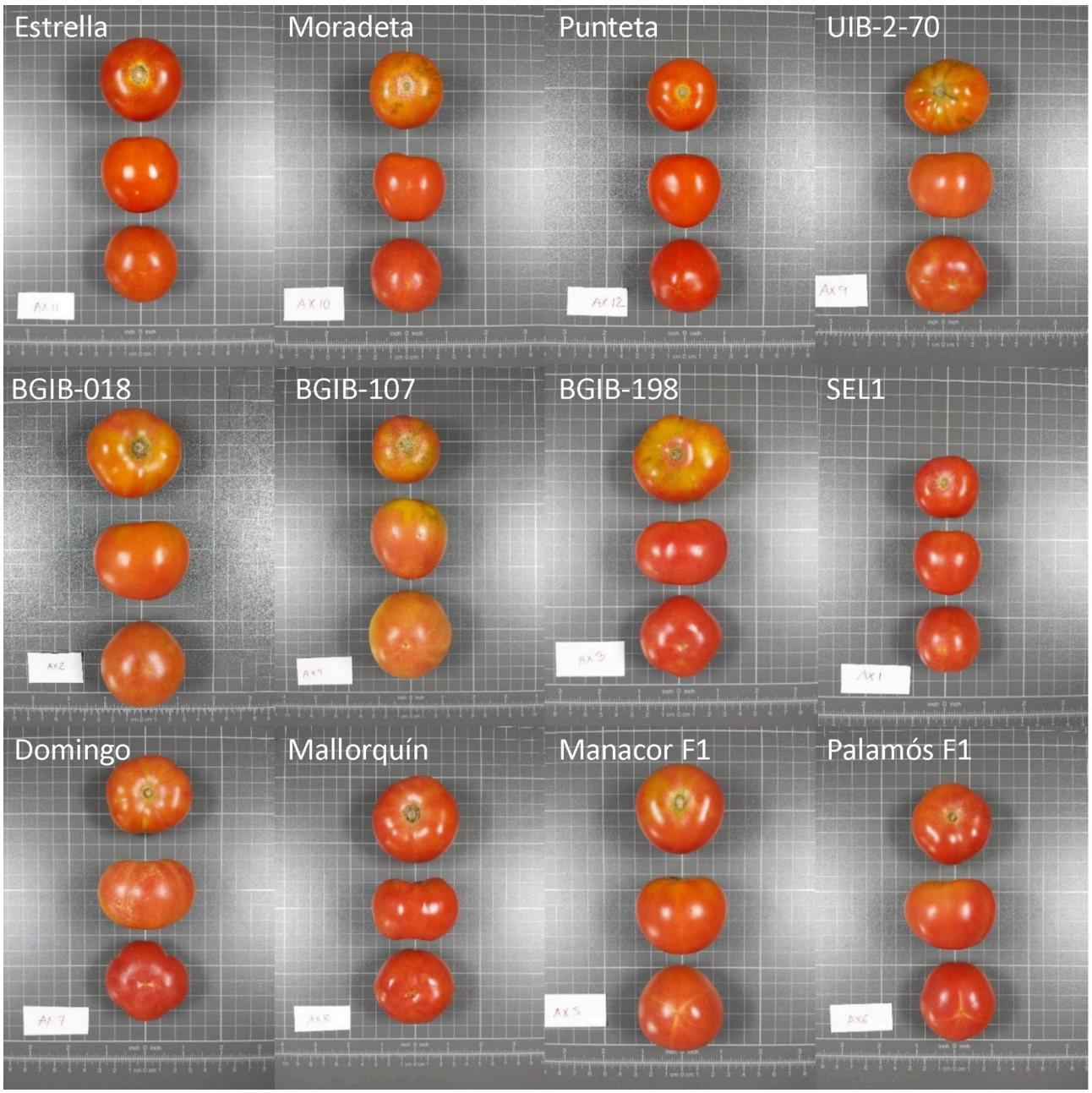

FIGURE 1 | Fruits of the 12 long shelf-life tomato varieties used for the characterization using morphological, agronomic, chemical properties, and chemical composition descriptors. Varieties "Estrella," "Moradeta," and "Punteta" correspond to landraces used for the production of the Valencian Community (Spain) Quality Mark "Tomata de Penjar"; variety "UIB-2-70" is the type landrace for the conservation variety "Tomàtiga de Ramellet" from Majorca Island (Spain); varieties "BGIB-018," "BGIB-107," "BGIB-198" correspond to the "Tomàtiga de Ramellet" highly variable landrace from Majorca Island; variety "SEL1" is a selection used for greenhouse cultivation in the Almería province (Spain), "Domingo," and "Mallorquín" are commercial varieties corresponding to selections from Semillas Batlle (Molins de Rei, Barcelona, Spain); and "Palamós F1" and "Manacor F1" are two commercial long shelf-life hybrids from Semillas Fitó (Barcelona, Spain). The grid cells in the pictures measure 1 × 1 cm.

applied 2 weeks after transplant and 0.042 kg/m^2 of fertilizer containing 15% N, 5% P_2O_5, and 30% K_2O was applied after fruits of the second or third trusses were in the cell expansion fruit development stage. For the OF trial, this makes an average amount of N, P, and K macroelements supplied with fertilizers of 28.9 g/plant of N, 11.6 g/plant of P, and 17.3 g/plant of K.

The plastic greenhouse used for the evaluation was also located in Alcalà de Xivert at a distance of 3 km from the open field plot. The greenhouse was of the multispan type and each

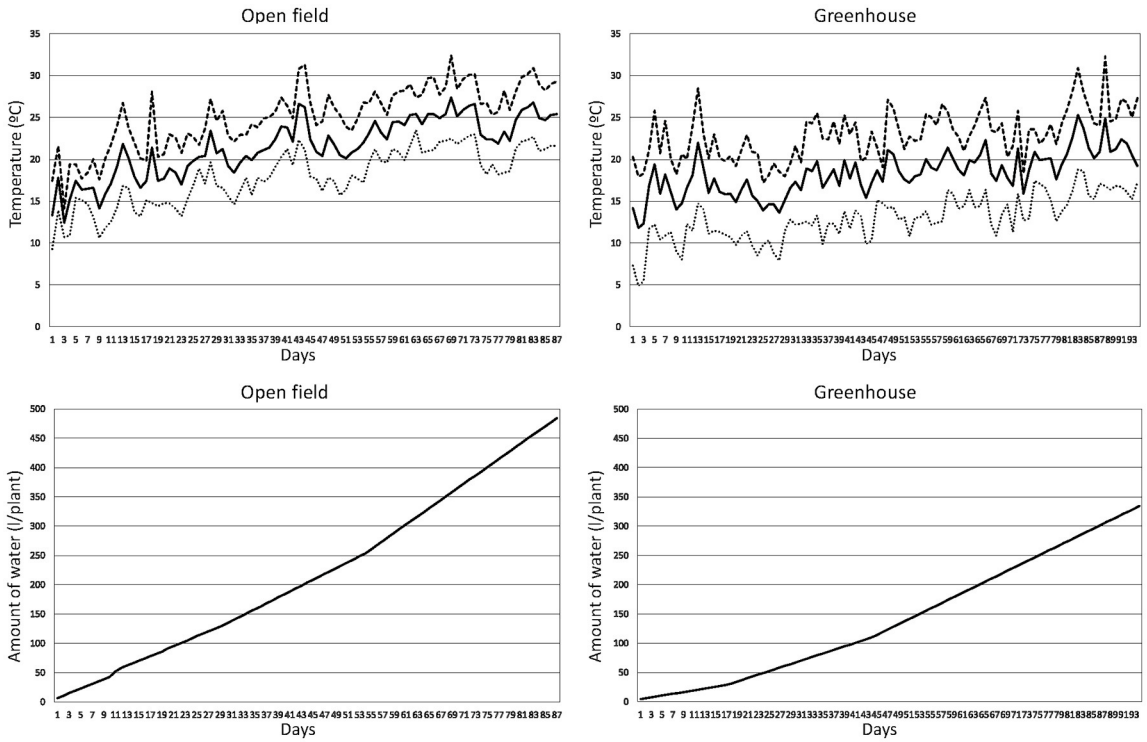

FIGURE 2 | Time course of temperatures and accumulated amounts of water per plant for the open field and greenhouse experiments with 12 long shelf-life tomato varieties. The graphs represent the daily minimum (dotted line), maximum (dashed line), and average (solid line) temperatures since the start of the experiments, which were the 29 April 2016 (open field experiment) and 19 February 2016 (greenhouse experiment), as well as the accumulated amounts of water per plant provided through the irrigation system (plus the rainfall water in the open field experiment).

module had a size of 52 × 8 m. The ceiling and laterals were covered with Celloflex 4 TT (Riviera Blumen, Puerto Lumbreras, Spain) multilayer polyethylene plastic. This greenhouse had automated cenital openings as well as manually operated lateral openings. Plants were distributed using the same plant density than for OF. Plants were trellised using vertical strings, pruned to remove side shoots. Irrigation was provided using a drip irrigation system like for OF using a total volume of 955.7 mm (334.5 l/plant; **Figure 2**). After an initial watering of 4.5 l/plant just after the transplant, 1.5 l/plant were applied daily until the day 17 after transplant, followed by 3 l/plant per day days until the day 43 after transplant, and finally 4.5 l/plant per day until the day 93 (1 day before the end of the experiment). The fertilization was the same than for OF, except that poultry manure was applied at a rate of 2.00 kg/m^2. For the GH trial, this makes an average amount of N, P, and K macroelements supplied with fertilizers of 21.8 g/plant of N, 8.6 g/plant of P, and 13.7 g/plant of K.

Preventive phytosanitary treatments were performed against whiteflies and *Tuta absoluta* with imidacloprid and emamectin in both OF and GH conditions, and weeds were removed with a hoe. Fruits of both trials were harvested at the red maturity stage (i.e., when fruits have between 60 and 90% of the skin with the typical fully ripe color of each accession) according to the scale defined by Yamaguchi (1983). Fruits used for morphometric and chemical measurements and analyses were harvested individually in a single day for each of the accessions when sufficient amounts of fruits at the appropriate red maturity stage were available in the plants.

Characterization

Varieties were characterized using 52 descriptors, including morphological (34), agronomic (6), physico-chemical properties (6), and chemical composition (6) traits. Morphological and agronomic descriptors were measured on a plant basis ($n = 10$). The morphological descriptors were quantitative (6), meristic (2), based a quantitative scale (19), or dichotomic (7) and corresponded to IPGRI (1996) tomato characterization descriptors (**Table 1**). The agronomic descriptors considered were: fruit weight (g); fruit shape (ratio length/width) obtained from IPGRI descriptors Fruit length and Fruit width; fruit firmness (Shore A standard units) measured in two opposite sides in the mid-part of the fruit between the proximal and distal ends of the fruit using a 53215 Fruit Hardness Tester (TR Turoni srl, Forli, Italy); color difference with true red obtained using the formula $[(L^*\text{-}50)^2+(a^*\text{-}60)^2+b^{*2}]^{0.5}$ from CIELAB fruit color parameters L*, a*, and b* measured in the central part of the fruit at a mid-distance between the distal and proximal parts using a CR-300 (Minolta, Osaka, Japan) chromameter; yield (kg/plant); and, daily moisture loss (%) by measuring the fruit weight at harvest and after storage for 30 days at room temperature of a sample of 10 fruits per plant and calculating the average daily loss. For descriptors involving measurements of fruits, 10 fruits

TABLE 1 | Morphological descriptors used for the characterization of 12 long shelf-life tomato varieties in two environments. Full details of the descriptors used can be consulted elsewhere (IPGRI, 1996).

Descriptors	IPGRI descriptor code	Units/scale
Plant growth type	7.1.2.1	1 = Dwarf; 4 = Indeterminate
Plant size	7.1.2.2	3 = Small; 7 = Large
Stem pubescence intensity	7.1.2.4	3 = Sparse; 7 = Dense
Foliage density	7.1.2.6	3 = Sparse; 7 = Dense
Number of leaves under 1st inflorescence	7.1.2.7	–
Leaf attitude	7.1.2.8	3 = Semi-erect; 7 = Dropping
Degree of leaf dissection	7.1.2.10	3 = Low; 7 = High
Anthocyanin coloration of leaf veins	7.1.2.11	1 = Obscure vein; 2 = Clear (normal)
Inflorescence type	7.2.1.1	1 = Generally uniparous; 3 = Generally multiparous
Number of flowers per inflorescence	8.1.5	–
Corolla blossom type	7.2.1.3	1 = Closed; 2 = Open
Style position	7.2.1.7	1 = Inserted; 4 = Highly exserted
Style shape	7.2.1.8	1 = Simple; 3 = Divided
Style hairiness	7.2.1.9	0 = Absent; 1 = Present
Dehiscence	7.2.1.11	1 = Poricidal; 2 = Longitudinal
Exterior color of immature fruit	7.2.2.1	1 = Greenish-white; 9 = Very dark green
Presence of green (shoulder) trips on the fruit	7.2.2.2	0 = Absent; 1 = Present
Intensity of greenback (shoulder)	7.2.2.3	3 = Slight; 7 = Strong
Fruit pubescence	7.2.2.4	3 = Sparse; 7 = Dense
Fruit size homogeneity	7.2.2.7	3 = Low; 7 = High
Fruit length	7.2.2.9	mm
Fruit width	7.2.2.10	mm
Easiness of fruit to detach from pedicel	7.2.2.15	3 = Easy; 7 = Difficult
Fruit shoulder shape	7.2.2.16	1 = Flat; 7 = Strongly depressed
Pedicel length	7.2.2.17	cm
Pedicel length from abscission layer	7.2.2.18	cm
Presence/absence of jointless pedicel	7.2.2.19	0 = Absent; 1 = Present
Width of pedicel scar	7.2.2.20	mm
Size of corky area around pedicel scar	7.2.2.21	mm
Skin color of ripe fruit	7.2.2.23	1 = Colorless; 2 = Yellow
Fruit blossom end shape	7.2.2.33	1 = Indented; 3 = Pointed
Radial cracking[a]	8.2.3	1 = Corky lines; 7 = Severe
Concentric cracking[a]	8.2.4	1 = Corky lines; 7 = Severe
Fruit fasciation	8.2.5	3 = Slight; 7 = Severe

[a] *Values of 0 were assigned for these descriptors when cracking was not observed.*

per plant were measured and values obtained for individual fruits were used to calculate the average value for each individual plant.

The chemical properties and chemical composition descriptors were measured on six samples ($n = 6$) taken from the bulked harvest of all plants, with at least five fruits per sample. Samples were squeezed with a domestic juice extractor and two aliquots were obtained: one for immediate determination of several traits and another one was frozen in liquid N_2 and stored at -80°C until used for the other traits.

Chemical properties measured were: dry matter (%) by drying at 105°C until constant weight; soluble solids (SS; %) by refractometry; pH with a pHmeter; titratable acid (TA; %) by titration of diluted juice (1:5) with 0.5 N NaOH to pH 8.1 and expressed as citric acid percentage; taste index (TI) by applying the formula $TI = TA + (SS/(20 \times TA))$ according to Navez et al. (1999); and, antioxidant activity (mM TE/g), measured using the colourimetric DPPH assay and expressed as Trolox equivalents (TE). All chemical properties were determined in the immediate analysis aliquot, with the exception of antioxidant activity, which was measured in the frozen aliquot. Chemical composition traits evaluated were the contents in: glucose (g/kg) and fructose (g/kg) measured using the D-Fructose/D-Glucose Assay Kit (Megazyme International Ltd., Wicklow, Ireland); citric acid using the CI9920 enzymatic kit (BEN S.r.l., Milan, Italy); ascorbic acid (mg/kg) by potentiometric titration with a Titrino 702 (Metrohm, Herisau, Switzerland) potentiometric titrator using a Metrohm 6.0420.100 combined Pt selective electrode and a 0.005 M chloramine solution as standard; lycopene (mg/kg) and β-carotene (mg/kg) by extraction overnight in darkness with ethanol:hexane (4:3 v/v), followed by separation of the hexane phase and determination of lycopene and β-carotene by UV/V spectrophotometry absorbance at 503 nm (lycopene) and 450 nm (β-carotene). All chemical composition analysis were performed in the frozen aliquot homogenate, except ascorbic acid, which was measured in the aliquot used for immediate analysis. Full details of the procedures for determining chemical properties and chemical composition traits are described elsewhere (Figàs et al., 2015b).

Data Analyses

Data for the morphological, agronomic, chemical properties, and chemical composition descriptors were subjected to a two factorial (variety and environment) analysis of variance (ANOVA) including the interaction among both main factors. The total sums of squares was partitioned in the sums of squares for variety, environment, variety × environment, and residual effects. For morphological descriptors, means and range were obtained for each environment. For agronomic, chemical properties, and chemical composition descriptors, the average value for each variety in each environment was calculated and the average standard error (SE) was obtained from the ANOVA analyses. Significance of differences among variety × environment combinations was evaluated using a Student-Newman-Keuls multiple range test at $P = 0.05$. A principal components analysis (PCA) was performed using pairwise Euclidean distances among variety means for each environment

using standardized data ($\mu = 0$; $\sigma = 1$) for the descriptors that were variable. All statistical analyses were performed using the Statgraphics Centurion XVI (StatPoint Technologies, Warrenton, VA, USA) software.

RESULTS

Analysis of Variance

Out of the 52 descriptors evaluated, six morphological descriptors were uniform across all varieties and environments. These descriptors and their states were: Corolla blossom type (1 = Closed); Style shape (1 = Simple); Dehiscence (2 = Longitudinal); Fruit pubescence (3 = Sparse); Presence/absence of jointless pedicel (0 = Absent); Concentric cracking (0 = No cracking). For the rest of descriptors, significant differences ($P < 0.05$) were found among varieties (**Table 2**). The percentage of sums of squares accounted for by the variety effect ranged between 8.8% (Radial cracking) and 100% (for Plant growth type and Skin color of ripe fruit). The variety effect was the greatest contributor to the sums of squares for most morphological descriptors. However, for the rest of descriptors the variety effect was only the greatest contributor for the agronomic descriptors Fruit weight and Fruit shape and for the chemical properties descriptors Dry matter and Soluble solids (**Table 2**). Significant differences among cultivation environments were found for 36 out of the 46 variable descriptors. Traits non-significant for the cultivation environment effect were six morphological ones as well as four related to chemical properties and composition (mostly related to acidity). The environmental effect was the main contributor to the sums of squares only for five descriptors, of which four were morphological (Foliage density, Leaf attitude, Pedicel length, and Width of pedicel scar) and the other one was the chemical composition descriptor Glucose (**Table 2**). The variety × environment interaction effect was significant for all variable descriptors, except for four (Plant growth type, Skin color of ripe fruit, pH, and Ascorbic acid). The variety × environment interaction was the greatest contributor to the sums of squares for three morphological descriptors (Number of leaves under 1st inflorescence, Intensity of greenback, and Fruit fasciation), while it had the same contribution than Variety for five other morphological descriptors (**Table 2**). The residual effect was the greatest contributor to the sums of squares for 14 descriptors, of which two were morphological (Pedicel length from abscission layer, and Radial cracking), four agronomic (all except Fruit weight and Fruit shape), three chemical properties (pH, Titratable acidity, and Antioxidant activity), and five chemical composition (all except Glucose) descriptors.

Variation for Morphological Descriptors

A wide range of variation among accessions for the 28 variable morphological descriptors was observed both under OF and GH environments (**Table 3**). For traits measured in a quantitative scale in most cases the range of variation covered an important part of the scale range. An exception was the Radial cracking in which a narrow range of variation was observed for this descriptor, as the incidence of cracking was very low (**Table 3**). For quantitative and meristic descriptors, a

TABLE 2 | Percentage of the total sums of squares for the effects of variety, environment, interaction between variety, and environment and residuals.

Descriptors	Sums of squares[a]			
	Variety	Environment	Variety × environment	Residual
MORPHOLOGICAL DESCRIPTORS				
Plant growth type	100.0***	0.0^{ns}	0.0^{ns}	0.0
Plant size	54.2***	24.1***	20.8***	0.9
Stem pubescence density	48.9***	2.1***	48.9***	0.0
Foliage density	22.8***	46.3***	30.9***	0.0
Number of leaves under 1st inflorescence	29.3***	0.2^{ns}	42.9***	27.5
Leaf attitude	21.4***	55.2***	21.4***	2.0
Degree of leaf dissection	43.4***	17.8***	38.3***	0.5
Anthocyanin coloration of leaf veins	47.8***	4.3***	47.8***	0.0
Inflorescence type	37.6***	21.8***	11.6***	29.0
Number of flowers per inflorescence	47.9***	12.8***	11.0***	28.4
Style position	57.4***	6.2***	14.9***	21.4
Style hairiness	42.9***	14.3***	42.9***	0.0
Exterior color of immature fruit	45.5***	5.0***	45.5***	4.0
Presence of green (shoulder) trips on the fruit	47.8***	4.3***	47.8***	0.0
Intensity of greenback (green shoulder)	40.0***	0.0^{ns}	58.1***	1.9
Fruit size homogeneity	39.9***	4.8***	38.1***	17.2
Fruit length	67.7***	0.1^{ns}	6.7***	25.5
Fruit width	59.6***	14.3***	6.8***	19.4
Easiness of fruit to detach from pedicel	49.5***	33.6***	16.8***	0.0
Fruit shoulder shape	46.5***	33.2***	7.4***	13.0
Pedicel length	15.9***	42.6***	7.4***	34.1
Pedicel length from abscission layer	49.2***	8.5***	8.3***	34.0
Width of pedicel scar	24.0***	42.6***	15.2***	18.2
Size of corky area around pedicel scar	23.7***	20.8***	13.6***	41.8
Skin color of ripe fruit	100.0***	0.0^{ns}	0.0^{ns}	0.0
Fruit blossom end shape	61.9***	5.1***	6.7***	26.3
Radial cracking	8.8*	0.6^{ns}	9.3*	81.4
Fruit fasciation	43.0***	2.4***	48.2***	6.4
AGRONOMIC DESCRIPTORS				
Fruit weight (g)	50.4***	22.4***	9.9***	17.3
Fruit shape	68.1***	12.9***	2.3*	16.7

(Continued)

TABLE 2 | Continued

Descriptors	Sums of squares[a]			
	Variety	Environment	Variety × Environment	Residual
Fruit firmness (Shore A standard units)	33.4***	12.0***	15.2***	39.4
Color difference with true red	24.2***	24.1***	6.5**	45.3
Yield (kg/plant)	18.0***	16.4***	12.2***	53.4
Daily moisture loss (%)	24.2***	24.1***	6.5**	45.3
CHEMICAL PROPERTIES DESCRIPTORS				
Dry matter (%)	49.3***	9.0***	15.8***	25.9
Soluble solids (%)	53.3***	2.1**	13.8***	30.9
pH	42.8***	1.2^{ns}	4.3^{ns}	51.6
Titratable acidity (%)	34.9***	0.1^{ns}	10.8*	54.3
Taste index	65.1***	0.1^{ns}	8.7***	26.1
Antioxidant activity (mM TE/g)	17.7***	8.3***	14.7**	59.3
CHEMICAL COMPOSITION DESCRIPTORS				
Glucose (g/kg)	11.3***	38.9***	12.9***	36.9
Fructose (g/kg)	17.6***	9.9***	12.1*	60.4
Citric acid (g/kg)	26.8***	0.0^{ns}	18.5***	54.7
Ascorbic acid (mg/kg)	40.3***	3.6**	5.1^{ns}	51.0
Lycopene (mg/kg)	16.0**	10.5***	14.2***	59.3
β-carotene (mg/kg)	13.0*	2.5*	18.3**	66.2

Descriptors include 46 morphological, agronomic, chemical properties, and chemical composition descriptors evaluated for which variation was observed in 12 long shelf-life tomato varieties grown in two environments (open field and greenhouse).
*[a],[ns], *, **, and ***Non-significant, or significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.*

considerable variation was also observed, with differences of over four-fold for the Number of flowers per inflorescence.

GH cultivation conditions resulted in relevant changes in the plant morphology compared to OF conditions, although the ranges of variation overlapped for all descriptors (**Table 3**). If we consider morphological traits for which there is a change of over 10% in GH with respect to OF, plants grown in GH had smaller plant size, less foliage density, leaves with a greater degree of dropping, and less divided, inflorescences with higher division and with more flowers, less exerted, and hairy styles, fruits less wide and easier to detach from pedicel, flatter fruit shoulder shape, longer pedicels, smaller pedicel scar, greater corky area around the pedicel scar, more pointed, and with greater fruit fasciation than those from OF (**Table 3**).

Variation for Agronomic Descriptors

Fruits from OF had a greater fruit weight (on average 40%) than those from GH, with a considerable variation among varieties, ranging between 60.2 g/fruit ("Moradeta") to 161.0 g/fruit ("Mallorquín") for OF and between 51.2 g/fruit ("Moradeta") and 89.0 g/fruit ("BGIB-018") for GH (**Table 4**). Fruits from OF were more flattened than those of GH, although under both conditions all varieties had a fruit length/width ratio below 1,

TABLE 3 | Means and range of variation for varietal means for 28 variable morphological descriptors in 12 long shelf-life tomato varieties grown in two environments (open field and greenhouse).

Descriptors	Open field		Greenhouse	
	Mean	Range	Mean[a]	Range
Plant growth type	3.83	2–4	3.83^{ns}	2–4
Plant size	6.50	5–7	5.58***	3–7
Stem pubescence density	5.00	5–5	4.83***	3–6
Foliage density	6.58	4–7	4.58***	3–7
Number of leaves under 1st inflorescence	6.99	6.3–7.3	7.13^{ns}	4.5–9.3
Leaf attitude	5.00	5–5	6.05***	5–7
Degree of leaf dissection	5.49	5–6	4.75***	3–6
Anthocyanin coloration of leaf veins	1.92	1–2	2.00***	2–2
Inflorescence type	1.45	1–2.5	2.16***	1–3
Number of flowers per inflorescence	5.99	4.5–11.2	8.32***	3.5–15.1
Style position	2.00	1.1–3.7	1.67***	1.0–2.4
Style hairiness	1.00	1–1	0.75***	0–1
Exterior color of immature fruit	3.18	3–5	3.00***	3–3
Presence of green (shoulder) trips on the fruit	0.92	0–1	1.00***	1–1
Intensity of greenback (green shoulder)	3.93	3–5	3.92^{ns}	3–6
Fruit size homogeneity	6.09	5–7	5.83***	5–6
Fruit length	4.33	3.70–4.96	4.30^{ns}	3.50–4.91
Fruit width	5.65	4.70–7.34	5.08***	4.08–5.87
Easiness of fruit to detach from pedicel	4.42	3–5	3.42***	3–5
Fruit shoulder shape	3.68	1.4–5	2.28***	1–3
Pedicel length	2.44	2.05–3.19	3.32***	2.92–3.88
Pedicel length from abscission layer	0.81	0.70–1.05	0.90***	0.70–1.04
Width of pedicel scar	0.72	0.48–1.23	0.41***	0.30–0.55
Size of corky area around pedicel scar	0.13	0.10–0.22	0.20***	0.08–0.30
Skin color of ripe fruit	1.17	1–2	1.17^{ns}	1–2
Fruit blossom end shape	1.41	1–3	1.70***	1–3
Radial cracking	0.01	0–0.1	0.05^{ns}	0–0.6
Fruit fasciation	0.10	0–0.7	0.36***	0–4

*Units or scale for each descriptor can be consulted in **Table 1**.*
*[a],[ns], and ***Differences between open field and greenhouse environments are non-significant, or significant at $p < 0.001$, respectively.*

except for variety "Punteta," with a value of 1.015 under GH conditions. Fruits from OF conditions were more firm than those of GH, with the exception of variety "SEL1" (**Table 4**). A smaller range of variation was observed for OF (between 46.8 Shore A standard units for "BGIB-018" and 67.4 Shore A standard units for "Punteta") than for GH (between 31.7 Shore A standard units for "Estrella" and 59.5 Shore A standard units for "Manacor F1"). Color difference with true red was of lower magnitude and the range of variation was narrower under OF than under GH (**Table 4**). Yield was, on average, 35% higher under OF than

TABLE 4 | Mean values for agronomic descriptors for 12 long shelf-life tomato varieties grown in open field (OF) and greenhouse (GH) environments.

	Fruit weight (g)[a]		Fruit shape (length/width ratio)		Fruit firmness (Shore a standard units)		Color difference with true red		Yield (kg/plant)		Daily moisture loss (%)	
Variety	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**
BGIB-018	97.9 hij	89.0 fgh	0.736 bcd	0.757 b–f	46.8 b–e	48.0 b–e	48.4 hi	45.8 e–h	4.58 fgh	3.05 a–e	0.183 a	0.286 a–f
BGIB-107	105.7 ijk	71.5 cde	0.876 ij	0.973 kl	60.9 hi	55.4 e–h	47.3 f–i	46.8 f–i	5.03 h	2.31 ab	0.175 a	0.243 a–e
BGIB-198	114.9 k	71.6 cde	0.688 ab	0.778 c–g	53.2 b–h	49.8 b–f	45.3 d–h	44.1 c–f	4.17 e–h	2.59 a–d	0.223 abc	0.404 g–j
Domingo	73.1 c–f	51.2 ab	0.667 a	0.717 abc	59.7 ghi	45.4 bc	49.7 i	45.8 e–h	3.47 b–g	4.30 e–h	0.196 ab	0.359 e–i
Estrella	84.4 e–h	59.6 bcd	0.788 d–g	0.846 ghi	54.8 d–h	31.7 a	46.9 f–i	47.2 f–i	4.32 e–h	2.44 abc	0.348 d–i	0.476 j
Mallorquín	161.0 l	87.1 e–h	0.657 a	0.803 d–h	52.3 b–h	44.4 b	44.7 c–g	42.1 bcd	4.77 gh	3.84 c–h	0.245 a–e	0.423 hij
Manacor F1	108.9 jk	75.7 d–g	0.872 hij	0.917 jk	59.6 ghi	59.5 ghi	48.0 ghi	46.8 f–i	4.63 fgh	3.91 d–h	0.275 a–e	0.443 ij
Moradeta	60.2 bcd	51.2 ab	0.827 f–i	0.915 jk	60.4 ghi	45.9 bcd	42.2 bcd	42.0 bc	3.74 b–h	2.85 a–e	0.304 b–g	0.334 c–h
Palamós F1	91.5 ghi	76.0 d–g	0.750 b–e	0.822 e–i	60.0 ghi	57.1 fgh	47.4 f–i	45.8 e–h	4.16 e–h	2.97 a–e	0.226 abc	0.391 f–j
Punteta	65.9 bcd	41.1 a	0.962 kl	1.015 l	67.4 i	58.2 fgh	40.7 b	37.4 a	3.15 a–e	1.84 a	0.302 b–g	0.323 c–h
SEL1	58.1 bc	52.4 ab	0.813 e–i	0.920 jk	49.7 b–f	54.0 c–h	42.9 b–e	42.2 bcd	2.92 a–e	2.32 ab	0.235 a–d	0.307 b–g
UIB-2-70	100.2 hij	73.3 c–f	0.694 ab	0.791 d–g	51.3 b–e	47.5 b–g	46.3 fgh	46.2 fgh	3.09 a–f	3.27 a–e	0.201 ab	0.255 a–e
SE	3.93		0.017		2.0		0.7		0.31		0.025	

The significance of the effects Variety, Environment (OF vs. GH), and Variety × Environment are presented in ***Table 2****. The standard error (SE) for pairwise comparison of the 24 combinations of Variety and Environment is provided.*

[a] *For each trait, mean values for combinations of Variety, and Environment separated by different letters are significant ($P < 0.05$) according to the Student-Newman-Keuls multiple range test. When a mean is followed by four or more letters, the range of letters is indicated.*

under GH. All varieties had a higher yield under OF than under GH, with the exception of "Domingo." Considerable variation among varieties was observed with ranges of variation between 2.92 kg/plant for "SEL1" and 5.03 kg/plant for "BGIB-107" in OF and between 1.84 kg/plant for "Punteta" and 4.0 kg/plant of "Domingo" in GH (**Table 4**). Fruits from GH had a higher (on average 41%) moisture loss during post-harvest than those from OF. Under both conditions the variety with lower daily moisture loss was "BGIB-107" with values of 0.175% and 0.243% under OF and GH, respectively, while the one with higher moisture loss was "Estrella" with values of 0.348 and 0.476% under OF and GH, respectively (**Table 4**).

Variation for Chemical Properties Descriptors

On average, fruits from GH cultivation had higher dry matter content (8.5%) than those from OF conditions, although for four varieties values were higher under OF conditions (**Table 5**). Values ranged between 5.61% for "BGIB-107" and 8.04% for "SEL1" under OF and between 5.46% for "BGIB-107" and 8.76% for "Punteta" under GH. Similarly, for soluble solids content fruits from GH had higher average contents than those of OF, although the differences were smaller (3.7%) than for dry matter content, and in five varieties the contents under OF were higher than those of GH (**Table 5**). As occurred for dry matter content, the variety with lowest values was "BGIB-107" with 5.23 and 5.05% under OF and GH, respectively, while the ones with highest values were "SEL1" under OF (6.98%) and "Moradeta" under GH (7.92%). Regarding pH, average differences among environments were non-significant, although for some varieties significant differences existed among environments (**Table 5**). The variety with lowest pH values was "BGIB-198" (4.08 in both environments) and the ones with highest values were "Estrella," "Mallorquín," and "Moradeta" under OF (4.38) and the latter under GH (4.39). As for pH, no significant differences were observed among environments for titratable acidity, although a considerable range of variation within each environment was observed, with values between 0.37% for "Mallorquín" and 0.62% for "BGIB-018" under OF and between 0.40% for "Estrella" and 0.58% for "Moradeta" under GH (**Table 5**). No differences among environments were observed for taste index among environments, although for some varieties significant differences were observed. In all cases the taste index value was above 1, with the lowest values observed in "BGIB-107" (1.02 in both environments) and the highest in "Punteta" (1.28 in OF and 1.32 in GH) (**Table 5**). The antioxidant activity was higher under GH (on average 25.6%) than under OF, although for three varieties it was higher under OF (**Table 5**). A wide range of variation was observed for antioxidant activity among varieties in both conditions, with values ranging between 0.57 mM TE/g for "Estrella" and 1.26 mM TE/g for "Palamós F1" under OF, and between 0.66 mM TE/g for "BGIB-018" and 1.70 mM TE/g for "Domingo" under GH (**Table 5**).

Variation for Chemical Composition Descriptors

Fruits from GH conditions had higher contents of glucose (on average 49%) than those from open field (**Table 6**). This higher content under GH conditions occurred in all varieties, except "Mallorquín." The range of variation under OF went from 11.6 g/kg in "BGIB-018" to 21.8 g/kg in "Mallorquín," while under GH went from 17.7 g/kg in "Domingo" to 31.5 g/kg in "Punteta." A

TABLE 5 | Mean values for chemical properties descriptors for 12 long shelf-life tomato varieties grown in open field (OF) and greenhouse (GH) environments.

	Dry matter (%)[a]		Soluble solids (%)		pH		Titratable acidity (%)		Taste index		Antioxidant activity (mM TE/g)	
Variety	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**
BGIB-018	7.34 d–h	6.78 c–f	6.58 ef	5.83 bcd	4.17 a–d	4.18 a–d	0.62 ef	0.50 a–f	1.18 cde	1.08 ab	1.12 abc	0.66 ab
BGIB-107	5.61 ab	5.46 a	5.23 ab	5.05 a	4.15 abc	4.18 a–d	0.50 a–f	0.44 a–e	1.02 a	1.02 a	0.77 ab	0.76 ab
BGIB-198	6.30 bc	8.51 ij	6.13 def	6.68 ef	4.08 a	4.08 a	0.60 def	0.65 f	1.11 bc	1.17 b–e	0.72 ab	1.01 ab
Domingo	6.19 abc	7.40 d–h	5.52 abc	6.35 def	4.26 a–d	4.32 bcd	0.42 abc	0.49 a–f	1.08 ab	1.14 bcd	0.89 ab	1.70 c
Estrella	7.16 c–h	7.08 c–g	6.37 def	5.97 bcd	4.38 cd	4.26 a–d	0.42 abc	0.40 ab	1.21 def	1.15 b–e	0.57 a	1.09 abc
Mallorquín	6.40 bcd	7.03 c–g	5.93 bcd	6.23 def	4.38 cd	4.33 bcd	0.37 a	0.45 a–e	1.17 b–e	1.15 b–e	0.81 ab	0.90 ab
Manacor F1	7.43 d–h	7.80 f–j	6.75 ef	6.72 ef	4.24 a–d	4.21 a–d	0.49 a–f	0.50 a–f	1.23 d–g	1.17 b–e	0.97 ab	1.18 abc
Moradeta	7.84 f–j	8.76 j	6.58 ef	7.92 h	4.38 cd	4.39 d	0.43 a–d	0.49 a–f	1.24 efg	1.30 gh	0.82 ab	1.18 abc
Palamós F1	6.65 cde	8.17 hij	6.27 def	6.50 ef	4.25 a–d	4.11 ab	0.48 a–f	0.58 b–f	1.14 bcd	1.14 bcd	1.26 abc	1.38 bc
Punteta	7.47 e–h	8.57 ij	6.78 ef	7.82 h	4.34 cd	4.33 bcd	0.40 a	0.45 a–e	1.28 fgh	1.32 h	0.72 ab	1.30 abc
SEL1	8.04 g–j	7.75 f–i	6.98 f	7.00 f	4.30 a–d	4.25 a–d	0.55 c–f	0.45 a–e	1.22 d–g	1.23 d–g	1.18 abc	1.15 abc
UIB-2-70	6.81 c–f	7.12 c–g	6.32 def	6.13 def	4.18 a–d	4.10 a	0.60	0.54	1.17	1.11 bc	0.91 ab	1.29 abc
SE	0.23		0.20		0.05		0.04		0.02		0.14	

The significance of the effects Variety, Environment (OF vs. GH), and Variety × Environment are presented in ***Table 2****. The standard error (SE) for pairwise comparison of the 24 combinations of variety and environment is provided.*

[a]For each trait, mean values for combinations of Variety, and Environment separated by different letters are significant ($P < 0.05$) according to the Student-Newman-Keuls multiple range test. When a mean is followed by four or more letters, the range of letters is indicated.

similar situation to that of glucose occurred for fructose content, with higher values (37.9% on average) under GH conditions, except for "Mallorquín." A wide variation was observed among varieties for fructose content, in particular under OF, with values ranging from 4.2 g/kg for "Estrella" to 19.7 g/kg for "Moradeta," while for GH values ranged between 13.2 g/kg for "Palamós F1" and 22.7 g/kg for "BGIB-018" (**Table 6**). Differences among environments for average values of citric acid content were non-significant, although many differences among environments were observed for individual varieties. In this respect, the ranges of variation under OF went from 1.53 g/kg in "Mallorquín" to 5.82 g/kg in "SEL1," while under GH went from 2.31 g/kg in "Domingo" to 9.46 g/kg in "BGIB-198" (**Table 6**). Ascorbic acid content was higher under GH than under OF (on average 4.4%), although for three varieties, values were higher under OF. The variety with lowest values under both conditions was "BGIB-107" with values of 277 mg/kg and 301 mg/kg under OF and GH, respectively, while the one with highest values was "Punteta," with values of 393 and 420 mg/kg under OF and GH, respectively (**Table 6**). Lycopene contents were, on average higher (67.4%) under OF than under GH, although for "Palamós F1" and "Punteta," higher values were obtained under GH. Considerable variation was observed for lycopene content in both environments with ranges between 13.8 mg/kg for "Estrella" and 70.7 mg/kg for "Moradeta" under OF, and between 9.5 mg/kg for "BGIB-107" and 25.7 mg/kg for "Punteta" under GH (**Table 6**). Similarly to lycopene, β-carotene contents were higher under OF (on average 17.2%) than under GH, except for three varieties. Ranges of variation for β-carotene varied between 6.8 mg/kg for "Manacor F1" and 13.0 mg/kg for "Domingo" under OF, and between 5.3 mg/kg for "BGIB-107" and 16.1 mg/kg for "Palamós F1" under OF (**Table 6**).

Principal Components Analysis

The first and second principal components in the PCA analysis accounted for 24.3 and 13.9% of the total variation, respectively (**Table 7**). The first principal component was positively correlated with several descriptors that had higher values under the OF environment, such as Foliage density, Style position, Style hairiness, Fruit width, Easiness of fruit to detach from pedicel, Fruit shoulder shape, Width of pedicel scar, and Yield (**Tables 3**, **4**), and negatively to descriptors that had lower values under OF environment such as Leaf attitude, Pedicel length, Pedicel length from abscission layer, Fruit blossom end shape, Fruit shape, Daily moisture loss, Dry matter, Soluble solids, Glucose, and Ascorbic acid, but also with Color difference with true red and Taste index (**Table 7**) which although had higher values under OF, the relative differences between both environments were small (**Tables 3**–**6**). The second principal component (**Table 7**) was positively correlated with several descriptors that had lower values under the OF environment such as Inflorescence type, Number of flowers per inflorescence, Pedicel length, Size of corky area around pedicel scar, and Fructose (**Tables 3**, **6**), and negatively with descriptors that had higher values under the OF environment such as Plant size, Degree of leaf dissection, Fruit size homogeneity, Fruit firmness, Lycopene, and β-carotene (**Tables 3**, **4**, **6**), but also to Plant growth type, Titratable acidity, Taste index, for which no significant differences existed between environments (**Tables 3**, **5**), or to Soluble solids, which although had higher values under GH the relative differences among environments were small (**Table 5**).

The projection of the 12 accessions grown in the OF and GH environments in the PCA plot clearly reveals a separation between both environments (**Figure 3**). Accessions grown under

TABLE 6 | Mean values for chemical composition descriptors for 12 long shelf-life tomato varieties grown in open field (OF) and greenhouse (GH) environments.

	Glucose (g/kg)[a]		Fructose (g/kg)		Citric acid (g/kg)		Ascorbic acid (mg/kg)		Lycopene (mg/kg)		β-carotene (mg/kg)	
Variety	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**	**OF**	**GH**
BGIB-018	11.6 a	22.9 def	11.6 ab	22.7 b	4.80 ab	3.55 a	348 bcd	345 bcd	36.6 a	15.4 a	11.6 abc	6.2 a
BGIB-107	13.4 ab	23.1 def	19.6 b	21.4 b	3.65 a	4.27 a	277 a	301 ab	38.5 a	9.5 a	8.7 abc	5.3 a
BGIB-198	17.7 a–e	27.3 fg	10.7 ab	18.0 b	5.06 ab	9.46 c	325 a–d	340 bcd	32.3 a	14.1 a	9.3 abc	8.5 abc
Domingo	14.8 abc	17.7 a–e	12.0 ab	16.0 ab	3.85 a	2.31 a	342 bcd	379 c–e	22.0 a	18.8 a	13.0 abc	9.6 abc
Estrella	13.4 ab	23.1 def	4.2 a	19.2 b	5.19 ab	3.06 a	336 bcd	369 c–e	13.8 a	12.6 a	11.6 abc	8.7 abc
Mallorquín	21.8 c–f	20.0 b–f	19.0 b	18.0 b	1.53 a	4.86 ab	367 b–e	377 c–e	26.1 a	19.6 a	7.7 ab	7.9 ab
Manacor F1	15.5 a–d	23.5 ef	9.3 ab	15.7 ab	5.05 ab	2.96 a	345 bcd	378 c–e	20.8 a	18.3 a	6.8 ab	8.7 abc
Moradeta	20.8 b–f	25.6 ef	19.7 b	15.0 ab	2.43 ab	2.44 a	320 abc	338 bcd	70.7 b	23.7 a	15.0 bc	7.7 ab
Palamós F1	17.5 a–e	23.7 ef	11.5 ab	13.2 ab	5.23 ab	8.78 bc	358 bcd	328 a–d	22.9 a	25.5 a	7.7 ab	16.1 c
Punteta	14.3 abc	31.5 g	4.7 a	13.9 ab	4.35 a	2.57 a	393 de	420 e	20.7 a	25.7 a	11.9 abc	9.9 abc
SEL1	17.2 a–e	24.2 ef	11.3 ab	14.1 ab	5.82 ab	2.88 a	343 bcd	372 c–e	32.2 a	22.4 a	10.7 abc	9.1 abc
UIB-2-70	14.9 abc	24.8 ef	15.1 ab	17.6 b	5.25 ab	6.09 ab	356 bcd	354 bcd	32.6 a	15.2 a	8.0 ab	7.6 ab
SE	1.7		2.6		0.92		13		6.6		1.6	

The significance of the effects Variety, Environment (OF vs. GH), and Variety × Environment are presented in ***Table 2****. The standard error (SE) for pairwise comparison of the 24 combinations of variety and environment is provided.*

[a]For each trait, mean values for combinations of Variety, and Environment separated by different letters are significant ($P < 0.05$) according to the Student-Newman-Keuls multiple range test. When a mean is followed by four or more letters, the range of letters is indicated.

OF conditions plot in a diagonal area of the graph that spans values going from a combination of intermediate values for the first component and low ones for the second component to a combination of high values for the first component and intermediate ones for the second component (**Figure 3**). Regarding accessions grown under GH conditions they also plot in a diagonal area of the graph with comparatively lower values for the first component and higher ones for the second. The PCA plot reveals that within each of the environments, accessions plot in analogous areas of the of the scatterplot. Accessions with lowest values for first and second components under OF conditions ("Punteta," "Moradeta," and "SEL1") are also the ones with lowest values for these components under GH conditions. The same occurs with accessions having highest values for both components, or intermediate values (**Figure 3**). Under both conditions accessions of the same origin plot in similar areas of the PCA graph. For example, in each of the environments, the three varieties from the "Tomata de Penjar" Quality Mark plot together and the same occurs for the four varieties from the Balearic Islands. Each of the two commercial selections plot together, and the same occurs for the two commercial hybrids (**Figure 3**).

DISCUSSION

Traditional long shelf-life tomato varieties carrying the *alc* mutation are well-adapted to open field cultivation and have specific characteristics that make them of special interest, like their tolerance to drought, extended post-harvest conservation period without refrigeration, and high contents in soluble solids (Mutschler et al., 1992; Conesa et al., 2014; Figàs et al., 2015b; Fullana-Pericàs et al., 2017, 2018). The two types of cultivation environment (OF and GH) are very different and our study was aimed at evaluating the performance of the tomatoes carrying the *alc* mutation under these two contrasting cultivation environments, which present many differences from the agronomic and management points of view, apart from taking place in different seasons of the year (Csizinsky, 2005; Peet and Welles, 2005; Figàs et al., 2018). As a consequence, the physiological mechanisms for growth and development processes acting in OF or GH conditions may be different, due to the great differences in temperature, solar radiation, wind, air humidity, and agricultural practices, among others (Tardieu, 2013).

Several descriptors that were uniform across the long shelf-life accessions and cultivation environments correspond to traits of taxonomic interest that distinguish tomato from some wild relatives, like the anther dehiscence type or the presence of fruit pubescence (Peralta et al., 2008), or traits that were introgressed from wild species into some modern tomato cultivars, such as the presence of jointless pedicel (Rick, 1967), or that appear as a physiological disorder caused by environmental factors or inappropriate cultivation practices, like the appearance of concentric cracking (Pascual et al., 2000). The two plant traits for which all the variation observed was caused by the enviromental effect (Plant growth type and Skin color of the ripe fruit) are monogenic and have a high penetration and expressivity (Carmen-Goren et al., 2003; Ballester et al., 2010), and confirm that there are *alc* long shelf-life varieties with determinate growth and that have colorless skin (i.e., resulting in pink colored fruits). The fact that the varietal effect was, in general, the largest one for morphological descriptors is important, as morphological descriptors used for characterizations should have a low environmental influence (Figàs et al., 2018). For the rest of descriptors, with the exception of fruit weight and fruit shape, which are largely genetically regulated (Panthee et al.,

TABLE 7 | Correlation coefficients between morphological, agronomic, chemical properties, and chemical composition descriptors and first and second principal components obtained from a multivariate principal components analysis.

Descriptors	First principal component	Second principal component
MORPHOLOGICAL DESCRIPTORS		
Plant growth type	−0.070	**−0.155**
Plant size	0.047	**−0.271**
Stem pubescence intensity	0.060	−0.013
Foliage density	**0.208**	−0.101
Number of leaves under 1st inflorescence	−0.096	−0.074
Leaf attitude	**−0.176**	0.128
Degree of leaf dissection	0.092	**−0.187**
Anthocyanin coloration of leaf veins	0.008	0.130
Inflorescence type	−0.064	**0.271**
Number of flowers per inflorescence	−0.058	**0.151**
Style position	**0.181**	0.093
Style hairiness	**0.198**	0.014
Exterior color of immature fruit	0.076	−0.037
Presence of green (shoulder) trips on the fruit	−0.035	0.104
Intensity of greenback (shoulder)	−0.002	0.056
Fruit size homogeneity	−0.050	**−0.216**
Fruit length	0.076	0.091
Fruit width	**0.244**	0.096
Easiness of fruit to detach from pedicel	**0.166**	−0.052
Fruit shoulder shape	**0.240**	−0.019
Pedicel length	**−0.223**	**0.175**
Pedicel length from abscission layer	**−0.220**	−0.061
Width of pedicel scar	**0.204**	−0.135
Size of corky area around pedicel scar	−0.061	**0.276**
Skin color of ripe fruit	−0.123	−0.127
Fruit blossom end shape	**−0.223**	−0.149
Radial cracking	−0.016	0.078
Fruit fasciation	−0.006	0.057
AGRONOMIC DESCRIPTORS		
Fruit weight (g)	**0.240**	0.038
Fruit shape	**−0.204**	−0.050
Fruit firmness (Shore A standard units)	0.079	**−0.221**
Color difference with true red	**−0.208**	0.087
Yield (kg/plant)	**0.219**	−0.052
Daily moisture loss (%)	**−0.208**	0.087
CHEMICAL PROPERTIES DESCRIPTORS		
Dry matter (%)	**−0.213**	−0.149
Soluble solids (%)	**−0.192**	**−0.222**
pH	−0.076	**−0.199**
Titratable acidity (%)	0.047	0.070
Taste index	**−0.163**	**−0.286**
Antioxidant activity (mM TE/g)	−0.132	0.022
Glucose (g/kg)	**−0.213**	0.124
Fructose (g/kg)	−0.028	**0.291**
Citric acid (g/kg)	0.039	0.045
Ascorbic acid (mg/kg)	**−0.152**	−0.092
Lycopene (mg/kg)	0.063	**−0.187**
β-carotene (mg/kg)	−0.015	**−0.198**
Variance explained (%)	24.3	13.9

Correlation values with absolute values ≥0.15 are presented in bold font.

2013; El-Gabry et al., 2014; Monforte et al., 2014), as well as for dry matter, soluble solids and taste index, the cultivation environment, variety × environment, or residual effects were the most important contributors. In other works, it has been found that environmental effects together with their interaction with variety have a large effect on these traits in tomato (Kuti and Konuru, 2005; Ortiz et al., 2007; Roselló et al., 2010; Adalid et al., 2012; Panthee et al., 2013; Figàs et al., 2018).

The characterization of the different types of descriptors revealed that a high diversity exists among the different materials of *alc* long shelf-life tomatoes, as for most of the descriptors a wide range of variation was observed. This is in agreement with other works (Cebolla-Cornejo et al., 2013; Bota et al., 2014; Mercati et al., 2015), which have found high diversity for morphological and agronomic descriptors, molecular markers, and chemical properties and composition traits in this varietal type. This suggests that the genetic background of *alc* tomatoes is large and that there are ample opportunities for selection within this varietal type.

The cultivation environment had a significant effect for many morphological traits, which was expected, due to the great differences among OF and GH environments for tomato cultivation (Csizinsky, 2005; Peet and Welles, 2005; Figàs et al., 2018). The highest yield under OF conditions probably is a consequence of the higher irradiation and higher temperatures during the summer season, which favor yield in tomato, compared to suboptimal conditions in the greenhouse. Although the yields of tomato in greenhouse are often higher than in the open field, due to a more controlled environment (Csizinsky, 2005; Peet and Welles, 2005), long shelf-life tomato landraces evolved and were selected for open field cultivation and need high temperatures and radiation for optimal flowering (Mercati et al., 2015; Fullana-Pericàs et al., 2018), which probably accounts for the generally lower yields under greenhouse. Some selections and commercial hybrids, like "Domingo," "Mallorquín," and "Manacor" gave the highest yield under GH conditions and therefore may be recommended under these conditions. Among the traits affected, fruits from GH cultivation were easier to detach from the pedicel than those from OF. This is important in this varietal group, as fruits are on many occasions threaded in strings (Casals et al., 2012; Mercati et al., 2015) and berries have to be firmly attached to the pedicel to avoid fruits breaking off

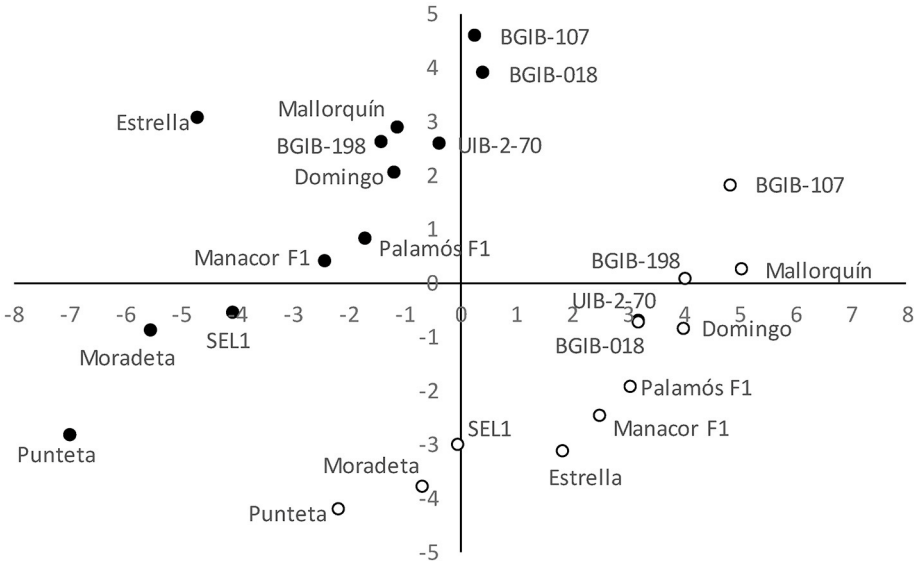

FIGURE 3 | First (*x*-axis) and second (y-axis) principal components scatterplot, based on 46 variable descriptors (28 morphological,6 agronomic, 6 chemical properties, and 6 chemical composition) in 12 long shelf-life tomato varieties grown under open field (OF; open circles) and greenhouse (GH; solid circles) environments. The first and second principal components account for 24.3 and 13.9% of the total variation. Each variety is indicated by its code.

to the ground. Therefore, varieties grown under GH conditions might be less appropriate for being threaded than those from the OF. Fruits from GH are more pointed than those of OF. Pointed fruits can be a disadvantage of GH cultivation, as this characteristic may increase the risk of fruit damage and bruising during harvesting and handling. Nonetheless, some long shelf-life varieties (like "Punteta") have pointed fruits. Greater fruit fasciation, an unfavorable trait, under GH might be caused by suboptimal environmental conditions resulting in fasciated flowers (Adams et al., 2001).

Fruits of *alc* long shelf-life tomato were relatively small when compared with other traditional tomato varieties (Bota et al., 2014; Figàs et al., 2015a) This is probably due to the negative correlation between fruit weight and post-harvest shelf-life in this varietal type (Casals et al., 2012). The fact that fruits from OF were larger than those of the GH could mean that the former could be less appropriate for post-harvest conservation. However, since OF fruits are generally more firm than those of GH indicates that the negative impact on post-harvest conservation of the larger fruit size of OF fruits can be compensated by their higher firmness. The fact that in most cases higher yields were obtained in the OF than under GH suggests a better adaptation of this varietal type to the traditional OF conditions, where it evolved and was selected (Casals et al., 2012; Cebolla-Cornejo et al., 2013; Bota et al., 2014; Mercati et al., 2015). Regarding post-harvest weight loss, it was low compared to standard tomato varieties (Javanmardi and Kubota, 2006; Pagno et al., 2017), and it was higher in fruits grown in GH, which is an indication of a better post-harvest performance of OF fruits.

The dry matter and soluble solids content was high compared to most standard tomato varieties (Rodríguez-Burruezo et al., 2005; Panthee et al., 2013; Figàs et al., 2015b). We found values of almost 8% for soluble solids in some varieties, suggesting that these materials could be a source of variation for breeding for dry matter and soluble solids content. Dry matter and soluble solids values have been higher under GH conditions, which probably is related to the reduced yield under these conditions. Several works indicate that in tomato there is a negative correlation between yield and soluble solids content (Dumas et al., 1994; Favati et al., 2009). pH and titratable acidity values were similar to those found in other works (Rodríguez-Burruezo et al., 2005; Panthee et al., 2013; Figàs et al., 2015b; Sánchez-González et al., 2015). In most *alc* long shelf-life varieties taste index was considerably higher than 1, which is considered as the optimal value for an equilibrated taste for salad tomato (Navez et al., 1999), and suggesting that fruits have an excess of soluble solids. Figàs et al. (2015b) also found that this varietal type, in general, has taste index values higher than 1. Traditional long shelf-life tomatoes carrying the *alc* mutation are generally used in a different way than the standard salad tomato (Casals et al., 2012; Romero del Castillo et al., 2014) and in most cases are used for rubbing into bread or used for cooking. Therefore, the different uses, compared to standard tomato used for being consumed raw in salads, probably have led to a selection of fruits with higher taste index in this varietal type. The fact that the antioxidant activity under GH conditions has been higher than under OF may be relevant for consumers (Diamanti et al., 2011), and the higher antioxidant activity might contribute to an extended post-harvest life (Zhang et al., 2013).

The levels of the chemical compounds analyzed here are similar to those obtained in other works for tomato in general (Rodríguez-Burruezo et al., 2005; Galiana-Balaguer et al., 2006; Panthee et al., 2013; Figàs et al., 2015b; Sánchez-González et al., 2015) and for this particular varietal type (Casals Missio et al., 2015; Figàs et al., 2015b), and reveal a considerable variation in the materials evaluated. As occurred for dry matter and soluble

solids content, the average glucose and fructose levels were higher under GH conditions, which was expected, as sugars are a major constituent of soluble solids in tomato (Beckles, 2012; Figàs et al., 2015b). In the same way, as observed for titratable acidity, no differences in average values were observed for citric acid, the major organic acid in tomato (Galiana-Balaguer et al., 2006). Like antioxidant activity, ascorbic acid content was higher under GH conditions, although similarly to what was found for cherry tomatoes, lycopene levels were higher under OF conditions (Kuti and Konuru, 2005). Given the much higher levels of ascorbic acid than those of carotenoids, our results provide an indication that in tomato ascorbic acid may have a greater contribution than lycopene to the total antioxidant activity of Mediterranean traditional long shelf-life tomato varieties (Cano et al., 2003; Sánchez-Moreno et al., 2006; Figàs et al., 2015b). The fact that the norm of reaction for antioxidant compounds against the cultivation environment of the varieties tested was very different, so that some varieties had higher levels of the antioxidant compounds under GH than under OF, indicates that the G × E interaction can be exploited for long shelf-life materials with higher levels of antioxidants in either OF or GH conditions.

The PCA analysis clearly separated the combinations of variety and cultivation environment according to the cultivation environments. In a former work (Figàs et al., 2018), in which several varietal types were evaluated, we found that the PCA grouped the accessions according to varietal group and not to cultivation environment. However, within varietal group such distinction was unclear (Figàs et al., 2018). In our case, in which all materials belong to a single cultivar group, the clear separation for environment in the PCA indicates a major impact of the cultivation environment (open field vs. greenhouse) on characteristics of the plants and fruits of this varietal type (Csizinsky, 2005; Peet and Welles, 2005; Figàs et al., 2018). However, it is evident from the PCA that the distribution of accessions under OF or GH conditions follow a similar pattern indicating a good correlation of the global characteristics of individual varieties in different environments. The fact that individual varieties from each origin or varietal type cluster in the same area of the plot relative to other varieties in both OF and GH conditions reveal that a phenotypic differentiation may exist within this varietal group, which may be exploited for selection and breeding (Panthee et al., 2013; Scott et al., 2013). Importantly, the commercial selections and hybrids carrying the *alc* allele are not in the extremes of distribution of the PCA scatterplots for either OF or GH, revealing that they have similar characteristics to those of the landraces.

As observed in other works (Casals et al., 2012; Cebolla-Cornejo et al., 2013; Bota et al., 2014; Figàs et al., 2015a,b; Mercati et al., 2015), our results reveal that a large diversity exists in the traditional long shelf-life tomato varietal group characterized by carrying the *alc* allele, with the largest diversity being present in the landraces. Compared to the traditional OF cultivation of the landraces of this varietal group, cultivation under greenhouse had a high impact on morphological, agronomic, chemical properties and chemical composition. Generally GH cultivation had a negative impact on some morphological traits, like a greater easiness of fruit to detach from pedicel, which is important for the traditional threading of the fruits in strings for hanging (Casals et al., 2012), in productive traits (e.g., lower yield and firmness and higher post-harvest loss), and in lycopene and β-carotene contents. However, it also increased dry matter, soluble solids, antioxidant activity, and glucose, fructose, and ascorbic acid contents. Although large G × E interaction could be exploited for selection for adaptation to greenhouse of this varietal type (Scott et al., 2013), our results suggest that specific breeding programmes for selecting long shelf-life materials carrying the *alc* mutation of the traditional "de colgar," "de penjar," "de ramellet," or "da serbo" specifically adapted to greenhouse cultivation are needed. In this respect, the diversity present in the landraces will be of great relevance for developing this new generation of varieties. Until these varieties are obtained, the evaluation of landraces and commercial selections and hybrids may allow identifying materials with better characteristics for greenhouse cultivation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JP, MR, and SS planned the study. JP, MP, and SS supervised the research. MF, LP-D, CC, MG-M, and ES performed the morphological, agronomic, and chemical properties characterization. MR, MG-M, and ES performed the chemical composition characterization. MF, CC, and ES supervised the crops. MF and MG-C curated the data. LP-D, ER, and MP performed the statistical analyses. MF, JP, MP, and SS drafted the manuscript.

FUNDING

This work was supported by Associació de Productors i Comercialitzadors de la Tomata de Penjar d'Alcalà de Xivert. Funding was also received from the TRADITOM (Traditional tomato varieties and cultural practices: a case for agricultural diversification with impact on food security and health of European population), G2P-SOL (Linking genetic resources, genomes, and phenotypes of Solanaceous crops) and BRESOV (Breeding for resilient, efficient, and sustainable organic vegetable production) projects. TRADITOM, G2P-SOL, and BRESOV projects have received funding from the European Union's Horizon 2020 research and innovation programme under grant agreements 634561 (TRADITOM), 677379 (G2P-SOL), and 774244 (BRESOV). ER is grateful to the Spanish Ministerio de Economía, Industria y Competitividad for a pre-doctoral grant (BES-2016-077482). MP is grateful to Spanish Ministerio de Economía, Industria y Competitividad for a post-doctoral grant within the Juan de la Cierva programme (FCJI-2015-24835) and to Generalitat Valenciana and Fondo Social Europeo for a post-doctoral contract (APOSTD/2018/014).

REFERENCES

Adalid, A. M., Roselló, S., Valcárcel, M., and Nuez, F. (2012). Analysis of the genetic control of β-carotene and L-ascorbic acid accumulation in an orange-brownish wild cherry tomato accession. *Euphytica* 184, 251–263. doi: 10.1007/s10681-011-0584-x

Adams, S. R., Cockshull, K. E., and Cove, C. R. J. (2001). Effect of the temperature on growth and development of tomato fruits. *Ann. Bot.* 88, 869–877. doi: 10.1006/anbo.2001.1524

Ballester, A. R., Molthoff, J., de Vos, R., Hekkert, B. L., Orzaez, D., Fernández-Moreno, J. P., et al. (2010). Biochemical and molecular analysis of pink tomatoes: deregulated expression of the gene encoding transcription factor S1MYB12 leads to pink tomato fruit color. *Plant Physiol.* 152, 71–84. doi: 10.1104/pp.109.147322

Barrett, D. M., Beaulieu, J. C., and Shewfelt, R. (2010). Color, flavor, texture, and nutritional quality of fresh-cut fruits and vegetables: desirable levels, instrumental and sensory measurement, and the effects of processing. *Crit. Rev. Food Sci. Nutr.* 50, 369–389. doi: 10.1080/10408391003626322

Beckles, D. M. (2012). Factors affecting the postharvest soluble solids and sugar content of tomato (*Solanum lycopersicum* L.) fruit. *Postharvest Biol. Technol.* 63, 129–140. doi: 10.1016/j.postharvbio.2011.05.016

Bota, J., Conesa, M. À., Ochogavia, J. M., Medrano, H., Francis, D. M., and Cifre, J. (2014). Characterization of a landrace collection for Tomàtiga de ramellet (*Solanum lycopersicum* L.) from the Balearic Islands. *Genet. Resour. Crop Evol.* 61, 1131–1146. doi: 10.1007/s10722-014-0096-3

Cano, A., Acosta, M., and Arnao, M. B. (2003). Hydrophilic and lipophilic antioxidant activity changes during on-vine ripening of tomatoes (*Lycopersicon esculentum* Mill.). *Postharvest Biol. Technol.* 28, 59–65. doi: 10.1016/S0925-5214(02)00141-2

Carmen-Goren, L., Liu, Y. S., Lifschitz, E., and Zamir, D. (2003). The *SELF-PRUNING* gene family in tomato. *Plant Mol. Biol.* 52, 1215–1222. doi: 10.1023/B:PLAN0000004333.96451.11

Casals Missio, J., Marti Renau, R., Casañas Artigas, F., and Cebolla Cornejo, J. (2015). Sugar-and-acid profile of Penjar tomatoes and its evolution during storage. *Sci. Agric.* 72, 314–321. doi: 10.1590/0103-9016-2014-0311

Casals, J., Cebolla-Cornejo, J., Roselló, S., Beltrán, J., Casañas, F., and Nuez, F. (2011). Long-term postharvest evolution of tomatoes with the alcobaça (*alc*) mutation. *Eur. Foor Res. Technol.* 233, 331–342. doi: 10.1007/s00217-011-1517-6

Casals, J., Pascual, L., Cañizares, J., Cebolla-Cornejo, J., Casañas, F., and Nuez, F. (2012). Genetic basis of long shelf life and variability into Penjar tomato. *Genet. Resour. Crop Evol.* 59, 219–229. doi: 10.1007/s10722-011-9677-6

Cebolla-Cornejo, J., Roselló, S., and Nuez, F. (2013). Phenotypic and genetic diversity of Spanish tomato landraces. *Sci. Hort.* 162, 150–164. doi: 10.1016/j.scienta.2013.07.044

Conesa, M. À., Galmés, J., Ochogavía, J. M., March, J., Jaume, J., Martorell, A., et al. (2014). The postharvest tomato fruit quality of long shelf-life Mediterranean landraces is substantially influenced by irrigation regimes. *Postharvest Biol. Technol.* 93, 114–121. doi: 10.1016/j.postharvbio.2014.02.014

Cortés-Olmos, C., Valcárcel, J. V., Roselló, J., Díez, M. J., and Cebolla-Cornejo, J. (2015). Traditional Eastern Spanish varieties of tomato. *Sci. Agric.* 5, 420–431. doi: 10.1590/0103-9016-2014-0322

Csizinsky, A. A. (2005). "Production in the open field," in *Tomatoes*, ed E. Heuvelink (Wallingford, UK: CABI), 237–256.

Diamanti, J., Battino, M., and Mezzetti, B. (2011). "Breeding for fruit nutritional and nutraceutical quality," in *Breeding for Fruit Quality*, eds M. A. Jenks and P. J. Bebeli (Hoboken, NJ: John Wiley & Sons), 61–80. doi: 10.1002/9780470959350.ch3

Dumas, Y., Leoni, C., Portas, C. A. M., and Bièche, B. (1994). Influence of water and nitrogen availability on yield and quality of processing tomato in the European Union countries. *Acta Hort.* 376, 185–192. doi: 10.17660/ActaHortic.1994.376.23

El-Gabry, M. A. H., Solieman, T. I. H., and Abido, A. I. A. (2014). Combining ability and heritability of some tomato (*Solanum lycopersicum* L.) cultivars. *Sci. Hort.* 167, 153–157. doi: 10.1016/j.scienta.2014.01.010

Fairchild, D. (1927). The tomato terraces of Bañalbufar: an agricultural monopoly built on a single variety of tomato. *Heredity* 18, 245–251. doi: 10.1093/oxfordjournals.jhered.a102861

Favati, A., Lovelli, S., Galgano, F., Miccolis, V., Di Tommaso, T., and Candido, V. (2009). Processing tomato quality as affected by irrigation scheduling. *Sci. Hort.* 122, 562–571. doi: 10.1016/j.scienta.2009.06.026

Figàs, M. R., Prohens, J., Casanova, C., Fernández-de-Córdova, P., and Soler, S. (2018). Variation of morphological descriptors for the evaluation of tomato germplasm and their stability across different growing conditions. *Sci. Hort.* 238, 107–115. doi: 10.1016/j.scienta.2018.04.039

Figàs, M. R., Prohens, J., Raigón, M. D., Fernández-de-Córdova, P., Fita, A., and Soler, S. (2015a). Characterization of a collection of local varieties of tomato (*Solanum lycopersicum* L.) using conventional descriptors and the high-throughput phenomics tool Tomato Analyzer. *Genet. Resour. Crop Evol.* 62, 189–204. doi: 10.1007/s10722-014-0142-1

Figàs, M. R., Prohens, J., Raigón, M. D., Fita, A., García-Martínez, M. D., Casanova, C., et al. (2015b). Characterization of composition traits related to organoleptic and functional quality for the differentiation, selection and enhancement of local varieties of tomato from different cultivar groups. *Food Chem.* 187, 517–524. doi: 10.1016/j.foodchem.2015.04.083

Fullana-Pericàs, M., Conesa, M. À., Soler, S., Ribas-Carbó, M., Granell, A., and Galmés, J. (2017). Variations of leaf morphology, photosynthetic traits and water-use efficiency in Western-Mediterranean tomato landraces. *Photosynthetica* 55, 121–133. doi: 10.1007/s11099-016-0653-4

Fullana-Pericàs, M., Ponce, J., Conesa, M. À., Juan, A., Ribas-Carbó, M., and Galmés, J. (2018). Changes in yield, growth and photosynthesis in a drought-adapted Mediterranean tomato landrace (*Solanum lycopersicum* 'Ramellet') when grafted onto commercial rootstocks and *Solanum pimpinellifolium*. *Sci. Hort.* 233, 70–77. doi: 10.1016/j.scienta.2018.01.045

Galiana-Balaguer, L., Roselló, S., and Nuez, F. (2006). Characterization and selection of balanced sources of variability for breeding tomato (*Lycopersicon*) internal quality. *Genet. Resour. Crop Evol.* 53, 907–923. doi: 10.1007/s10722-004-6696-6

Hounsome, N., Hounsome, B., Tomos, D., and Edwards-Jones, G. (2008). Plant metabolites and nutritional quality of vegetables. *J. Food Sci.* 73, R48–R65. doi: 10.1111/j.1750-3841.2008.00716.x

IPGRI (1996). *Descriptors for Tomato (Lycopersicon spp.)*. Rome: International Plant Genetic Resources Institute.

Javanmardi, J., and Kubota, C. (2006). Variation of lycopene, antioxidant activity, total soluble solids and weight loss of tomato during postharvest storage. *Postharvest Biol. Technol.* 41, 151–155. doi: 10.1016/j.postharvbio.2006.03.008

Kaushik, P., Andújar, I., Vilanova, S., Plazas, M., Gramazio, P., Herraiz, F. J., et al. (2015). Breeding vegetables with increased content in bioactive phenolic acids. *Molecules* 20, 18464–18481. doi: 10.3390/molecules201018464

Kuti, J. O., and Konuru, H. B. (2005). Effects of genotype and cultivation environment on lycopene content in red-ripe tomatoes. *J. Sci. Food Agric.* 85, 2021–2026. doi: 10.1002/jsfa.2205

Kyriacou, M. C., and Rouphael, Y. (2018). Towards a new definition of quality for fresh fruits and vegetables. *Sci. Hort.* 214, 463–469. doi: 10.1016/j.scienta.2017.09.046

Maamar, B., Maatoug, M., Iriti, M., Dellal, A., and Hammou, M. A. (2015). Physiological effects of ozone exposure on de Colgar and Rechaiga II tomato (*Solanum lycopersicon* L.) cultivars. *Environ. Sci. Pollut. Res.* 22, 12124–12132. doi: 10.1007/s11356-015-4490-y

Marín, J. (2015). *Portagrano: Vademecum de Semillas – Variedades Hortícolas*. El Ejido: José Marín Rodríguez.

Mercati, F., Longo, C., Poma, D., Aratini, F., Lupini, A., Mammano, M. M., et al. (2015). Genetic variation of an Italian long shelf-life tomato (*Solanum lycopersicon* L.) collection by using SSR and morphological traits. *Genet. Resour. Crop Evol.* 62, 721–732. doi: 10.1007/s10722-014-0191-5

Monforte, A. J., Diaz, A., Caño-Delgado, A., and van der Knaap, E. (2014). The genetic basis of fruit morphology in horticultural crops: lessons from tomato and melon. *J. Expt. Bot.* 65, 4625–4637. doi: 10.1093/jxb/eru017

Mutschler, M. A., Wolfe, D. W., Cobb, E. D., and Yourstone, K. S. (1992). Tomato fruit quality and shlef life in hybrids heterozygous for the *alc* ripening mutant. *HortScience* 27, 352–355.

Navez, B., Letard, M., Graselly, D., and Jost, J. (1999). Les critères de qualité de la tomate. *Infos-Ctifl* 155, 41–47.

Ortiz, R., Crossa, J., Vargas, M., and Izquierdo, J. (2007). Studying the effect of environmental variables on the genotype × environment interaction of tomato. *Euphytica* 153, 119–134. doi: 10.1007/s10681-006-9248-7

Pagno, C. E., Castagna, A., Trivellini, A., Mensuali-Sodi, A., Ranieri, A., Ferreira, E. A., et al. (2017). The nutraceutical quality of tomato fruit during domestic storage is affected by chitosan coating. *J. Food Process. Preserv.* 42:e13326. doi: 10.1111/jfpp.13326

Panthee, D. R., Labate, J. A., McGrath, M. T., Breksa, A. P., and Robertson, L. D. (2013). Genotype and environmental interaction for fruit quality traits in vintage tomato varieties. *Euphytica* 193, 169–182. doi: 10.1007/s10681-013-0895-1

Pascual, B., Maroto, J. V., Sanbautista, A., López-Galarza, S., and Alagarda, J. (2000). Influence of watering on the yield and cracking of cherry, fresh-market and processing tomatoes. *J. Hort. Sci. Biotechnol.* 75, 171–175. doi: 10.1080/14620316.2000.11511218

Peet, M. M., and Welles, G. (2005). "Greenhouse tomato production," in *Tomatoes*, ed E. Heuvelink (Wallingford, UK: CABI), 257–304.

Peralta, I. E., Spooner, D. M., and Knapp, S. (2008). Taxonomy of wild tomatoes and their relatives (*Solanum* sect. *Lycopersicoides*, sect. *Juglandifolia*, sect. *Lycopersicon*; Solanaceae). *Syst. Bot. Monogr.* 84, 1–186. doi: 10.2307/25027972

Rick, C. M. (1967). Fruit and pedicel characters derived from Galápagos tomatoes. *Econ. Bot.* 21:171–184. doi: 10.1007/BF02897867

Rodríguez-Burruezo, A., Prohens, J., Roselló, S., and Nuez, F. (2005). "Heirloom" varieties as sources of variation for the improvement of fruit quality in greenhouse-grown tomatoes. *J. Hort. Sci. Biotech.* 80, 453–460. doi: 10.1080/14620316.2005.11511959

Romero del Castillo, R., Puig-Rey, M., Biarnés, J., Vilaseca, H., Sim,ó, J., Plans, M., et al. (2014). Using trendsetting chefs to design new culinary preparations with the "Penjar" tomato. *J. Culin. Sci. Technol.* 12, 196–214. doi: 10.1080/15428052.2014.880099

Roselló, S., Adalid, A. M., Cebolla-Cornejo, J., and Nuez, F. (2010). Evaluation of the genotype, environment and their interaction on carotenoid and ascorbic acid accumulation in tomato germplasms. *J. Sci. Food Agric.* 91, 1014–1021. doi: 10.1002/jsfa.4276

Sánchez-González, M. J., Sánchez-Guerrero, M. C., Medrano, E., Porras, M. E., Baeza, E. J., and Lorenzo, P. (2015). Influence of pre-harvest factors on quality of a winter cycle, high commercial value, tomato cultivar. *Sci. Hort.* 189, 104–111. doi: 10.1016/j.scienta.2015.03.044

Sánchez-Moreno, C., Plaza, L., de Ancos, B., and Cano, M. P. (2006). Impact of high-pressure and traditional thermal processing of tomato puree on carotenoids, vitamin C and antioxidant activity. *J. Sci. Food Agric.* 86, 171–179. doi: 10.1002/jsfa.2321

Scott, J. W., Myers, J. R., Boches, P. S., Nichols, C. G., and Angell, F. F. (2013). "Classical genetics and traditional breeding," in *Genetics, Genomics and Breeding of Tomato*, eds B. E. Liedl, J. A. Labate, J. R. Stommel, A. Slade, and C. Kole (Boca Raton, FL: CRC Press), 37–73.

Tardieu, F. (2013). Plant response to environmental conditions: assessing potential production, water demand, and negative effects of water deficit. *Front. Physiol.* 4:17. doi: 10.13389/fphys.2013.00017

Tranchida-Lombardo, V., Cigliano, R. A., Anzar, I., Landi, S., Palombieri, S., Colantuono, C., et al. (2018). Whole-genome re-sequencing of two Italian tomato landraces reveals sequence variations in genes associated with stress tolerance, fruit quality and long shelf-life traits. *DNA Res.* 25, 149–160. doi: 10.1093/dnares/dsx045

Yamaguchi, M. (1983). "Solanaceous fruits: tomato, eggplant, pepper and others," in *World Vegetables*, ed M. Yamaguchi (New York, NY: AVI Books), 291–312. doi: 10.1007/978-94-011-7907-2_22

Zhang, Y., Butelli, E., De Stefano, R., Schoonbeek, H. J., Magusin, A., Pagliarani, C., et al. (2013). Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. *Curr. Biol.* 23, 1094–1100. doi: 10.1016/j.cub.2013.04.072

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 18 October 2018
doi: 10.3389/fpls.2018.01491

A Comparison of Landraces vs. Modern Varieties of Lettuce in Organic Farming During the Winter in the Mediterranean Area: An Approach Considering the Viewpoints of Breeders, Consumers, and Farmers

Joan Casals Missio[1,2], Ana Rivera[1], Maria Rosario Figàs[3], Cristina Casanova[3], Borja Camí[4], Salvador Soler[3] and Joan Simó[1,2]*

[1] Miquel Agustí Foundation, Campus del Baix Llobregat, Catalonia, Spain, [2] Department of Agri-Food Engineering and Biotechnology, BarcelonaTech, Campus del Baix Llobregat, Catalonia, Spain, [3] Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, Valencia, Spain, [4] Arrreu Tools for Agroecological Accompaniment, Barcelona, Spain

OPEN ACCESS

Edited by:
Lillian Barros, Polytechnic Institute of Bragança, Portugal

Reviewed by:
Francesco Di Gioia, Pennsylvania State University, United States
Nikos Tzortzakis, Cyprus University of Technology, Cyprus

***Correspondence:**
Joan Casals Missio
j.casalsmissio@gmail.com

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *05 July 2018*
Accepted: *25 September 2018*
Published: *18 October 2018*

Citation:
Missio JC, Rivera A, Figàs MR, Casanova C, Camí B, Soler S and Simó J (2018) A Comparison of Landraces vs. Modern Varieties of Lettuce in Organic Farming During the Winter in the Mediterranean Area: An Approach Considering the Viewpoints of Breeders, Consumers, and Farmers. Front. Plant Sci. 9:1491. doi: 10.3389/fpls.2018.01491

The interest of farmers in growing lettuce landraces is increasing, as landrace varieties prove particularly appealing to consumers striving to purchase natural, local, and high-quality produce. Although high genetic diversity exists in the landrace gene pool, this has scarcely been studied, thus hindering landrace utilization in agriculture. In this study, we analyzed the genetic diversity and the agronomic and quality traits of lettuce landraces in organic agrosystems, by characterizing 16 landraces and 16 modern varieties. We compared 29 morphological descriptors, and several traits relating to agronomic behavior (total and commercial weight, resistance to *Bremia lactucae*) and quality (color, chlorophyll, dry matter, and total sugars). Trials were conducted in two localities and managed following organic farming practices. Moreover, farmers and consumers participated in the phenotyping of accessions by scoring yield, resistance to *B. lactucae*, appearance, and taste acceptance. Results show that cultivar group, rather than the genetic origin (modern vs. landrace), is the major source of variation for all agronomic and quality traits. Batavia and Butterhead were highly homogeneous cultivar groups, while Cos accessions showed a much higher intra-varietal diversity. There was also a clear separation between modern and landrace varieties of Oak leaf. Fifteen out of the 16 evaluated landraces presented a high susceptibility to the particular *B. lactucae* race isolated from the experimental field - a new race not reported before. Breeding programs intended to introgress genetic resistance to this pathogen are a major priority to recover the cultivation of lettuce landraces. Principal component analysis (PCA), conducted on all quantitative data, showed a clear differentiation between modern varieties and landraces, mostly related to their commercial weight and susceptibility to *B. lactucae.* These seem the most important traits influencing farmer and consumer

evaluations. Farmers showed a high capacity for characterizing the samples and agreed with consumers when scoring for the external appearance. It is proposed that farmers and consumers should be included in the phenotyping platforms in future research projects aiming for recovery of landraces.

Keywords: agrobiodiversity, *Lactuca sativa* L., *Bremia lactucae*, participatory plant breeding, plant phenotyping

INTRODUCTION

Vegetable landraces (locally adapted, traditional plant varieties) have been generally displaced from market-driven production due to their lower yields, inferior pest and disease resistance, and poorer postharvest shelf life in comparison with modern varieties (van de Wouw et al., 2010). This has led to serious cultural and genetic erosion over the past 100 years (Negri, 2003; Hammer and Teklu, 2008). However, landraces are presently living a rebirth, driven by consumer demand for natural, local, and high-quality produce. New consumer groups, interested in purchasing quality foods linked to traditional and environmentally friendly labels, together with farmers concerned with the environmental and social impacts of food production, are rediscovering landraces as a source of value-added foods intrinsically associated with local production (Villa et al., 2005). Nevertheless, although significant efforts have been devoted in recent decades to collect and preserve landraces *ex situ* (Gepts, 2006), generally materials are stored in seed banks without any phenotypic information (Prada, 2009), thus hindering their utility to farmers. Therefore, it is of great importance to characterize these materials to make them available for commercial cultivation, and actualize their agronomic, sensory, and postharvest performances, to fit with current agriculture and consumption standards (Casañas et al., 2017). The classical approach for such characterization studies involves the phenotyping by research centers of the most important agronomic and quality traits, with the objective to describe yield performance and identify particular sensory or nutritional traits enhancing the distinctiveness of each variety. Nevertheless, to increase the worth of these studies to farmers, and include traits most relevant for consumers, the active integration of both of these groups in the phenotyping platform may offer a suitable alternative. This can be done through integration of sensory analysis (Tesfaye et al., 2013) and participatory plant breeding methodologies (Morris and Bellon, 2004) in a conjoint phenotyping platform with plant breeders.

The Iberian Peninsula is a hotspot for agrobiodiversity (Veteläinen and Maxted, 2009). Although for some crop species landraces are still present in the market [particularly for tomato (*Solanum lycopersicum* L.) and dry beans (*Phaseolus vulgaris* L.)], for other historically important crops, landraces are often enclosed in home gardens managed by old farmers (Casals et al., 2017). This is the case for lettuce (*Lactuca sativa* L.), an important leafy vegetable in European cuisine, which was domesticated in the eastern Mediterranean basin (Mou, 2008). Although it has great dietary and economic importance in Spain, the fourth greatest producing country in the world (Food and Agriculture Organization Corporate Statistical Database [FAOSTAT], 2016), and the richness of local cultivars have been preserved, landraces still remain marginal in the markets. In the area of study (Catalonia, NE Spain), several landraces were anciently appreciated, for instance, *cua d'oreneta* ("swallow tail"), *enciam del sucre* ("sugar lettuce"), *enciam negre* ("black lettuce"), or *enciam dels tres ulls* ("three eyed lettuce"). Most of these varieties remain cultivated in small areas, and others solely present in *ex situ* collections (Casals et al., 2017). To successfully recover the cultivation of lettuce landraces, there is a present need to investigate the genetic diversity at both phenotypic and molecular levels, which has been scarcely addressed in the scientific literature (Jansen et al., 2006; Vicente et al., 2008).

In contrast to other major crops, where significant increases in yield have been obtained by selecting for the harvested organ (seed, fruit, and tuber), higher lettuce biomass is not a trait generally present in the ideotypes of plant breeding programs (Still, 2007). For these species, the appearance of high-yielding modern varieties (i.e., producing a higher biomass per unit area of the harvested organ) seems not the principal factor driving the substitution of lettuce landraces, as has been the case for most other horticultural crops (van de Wouw et al., 2010). Other characteristics such as postharvest shelf life or resistance to pest and diseases have been more important in this process. Resistance to downy mildew (*Bremia lactucae* Regel) and lettuce aphid [*Nasonovia ribisnigri* (Mosley)] are currently the main characteristics driving lettuce breeding (Mou, 2008). Downy mildew is the most significant disease affecting lettuce, and the most efficient control strategy is the genetic resistance conferred by *Dm* genes (Michelmore and Wong, 2008). The gene-for-gene interaction between *L. sativa* and *B. lactucae*, and the pathogen variability, has led to continuous efforts of plant breeders to select for new resistance genes. So far, 28 *Dm* genes have been described, and modern lettuce varieties each carry a particular set of these genes (Parra et al., 2016). Usually farmers select the varieties to be cultivated based on the number of races for which one variety is resistant. Thus, the comparative lack of resistance to downy mildew in landraces (van Treuren et al., 2013) is the principal factor that has provoked their replacement by modern lettuce varieties. Other factors, such as cultivar diversification (some types are not present in the landrace gene pool), postharvest shelf life, and product standardization may also have had an important role.

Cultivation of lettuce is known to offer high profitability for farmers during the winter season (October-March) due to its resistance to cold temperatures, the minimal human labor needed during the crop cycle, and the lack of competence for agricultural land with other crops during this season. However, low temperatures and high humidity favor the incidence of

TABLE 1 | List of accessions characterized.

ID[1]	Variety name	Accession[2]	Type	Donor	Cultivar group[3]	Earliness (DAT)[4]	Resistances[5]
13	Negre	FMA/113	LR	FMA	Batavia	130-135	
	Carxofet	FMA/112	LR	FMA	Batavia	112-122	
	Meravella	FMA/99	LR	FMA	Batavia	133-140	
	Meravella d'hivern		LR	Plant nursery (Pastoret)	Batavia	130-140	
9	Carxofet	FMA/5	LR	FMA	Butterhead	116-130	
11	De primavera	FMA/87	LR	FMA	Butterhead	123-135	
	Carxofet		LR	Plant nursery (Pastoret)	Butterhead	107-122	
	Negre	FMA/253	LR	FMA	Cos	124-134	
	D'hivern	FMA/252	LR	FMA	Cos	121-129	
	Del terreno	FMA/134	LR	FMA	Cos	135-140	
15	Negre de reus		LR	Plant nursery (Pastoret)	Cos	130-140	
16	Negre de Vilafranca		LR	Plant nursery (Pastoret)	Cos	114-122	
14	Negre borratger	386/935	LR	SIGMA	Cos	128-135	
10	Cua d'oreneta		LR	Plant nursery (Pastoret)	Oak leaf	113-130	
12	Francès	219/855	LR	SIGMA	Oak leaf	125-140	
	Fulla de roure	60/387	LR	SIGMA	Oak leaf	125-140	
2	Carmen		MV	Gautier	Batavia	133-140	LMV: 1
5	Magenta		MV	Gautier	Batavia	126-140	16, 21, 23, 32/LMV: 1
7	Novelsky		MV	Rijk Zwaan	Batavia	140-150	Bl: 16-28, 30-32, Nr: 0
	Arena		MV	Vilmorin	Batavia	130-140	
8	Pomery		MV	Gautier	Butterhead	114-122	Bl: 16-32/Nr: 0/LMV: 1
4	Janique		MV	Nunhems	Butterhead	117-126	Bl: 16-30, 32/Nr: 0
1	Abago		MV	Rijk Zwaan	Butterhead	115-122	Bl: 16-31/Nr: 0/LMV: 1
	Amboise		MV	Gautier	Lollo	128-140	Bl: 16-27, 29, 30, 32/Nr: 0
	Rivero		MV	Clause	Oak leaf	121-135	Bl: 1-28, 28, Nr: 0
	Camarde		MV	Gautier	Oak leaf	118-122	Bl: 16-32/Nr: 0/LMV: 1
	Kiari		MV	Nunhems	Oak leaf	130-145	Bl: 16-32/Nr: 0/Fol: 1 HR
	Navara		MV	Nunhems	Oak leaf	126-135	Bl: 16-26, 28, 32/Nr: 0
3	Conuai		MV	Rijk Zwaan	Oak leaf	121-135	Bl: 16-32/Nr: 0/LMV: 1
	Rutilai		MV	Rijk Zwaan	Oak leaf	115-122	Bl: 16-32/Nr: 0/LMV: 1
6	Mathix		MV	Vitalis	Oak leaf	115-122	Bl: 16-32/Nr: 0/Pb
	Horix		MV	Vitalis	Oak leaf	108-122	Bl: 16-29, 32/Nr: 0/Pb

[1]*Accessions evaluated by consumers.*
[2]*For genotypes obtained from seed banks (FMA, SIGMA), accession number is provided.*
[3]*Cultivar group according to UPOV classification (International Union for the Protection of New Varieties of Plants [UPOV], 2016).*
[4]*Earliness: number of days after transplant (DAT) (50% of plants reached the commercial stage) measured in La Múnia (first value) and Benifallet (second value).*
[5]*Genetic resistances according to the information provided by seed companies.*
LR, landrace; MV, modern variety.

downy mildew (Mou, 2008), making cultivation of non-resistant lettuce varieties during this season extremely difficult. Farmers interested in distinguishing their products in the food market are embracing organic farming and landrace labels, and desire landraces that show good agronomic and quality characteristics under these conditions. The objective of this study was to evaluate the genetic diversity and describe the agronomic performance and quality characteristics of lettuce landraces in organic agrosystems. We evaluated 16 landraces and 16 modern varieties of lettuce by means of a multi-stakeholder approach, including the participation of farmers (through participatory plant-breeding protocols) and consumers (through sensory analysis). This type of complex phenotyping platform enabled description of the principal differences between landraces and modern varieties, and identification of the key factors driving both farmer and consumer preferences. Moreover, the *B. lactucae* race present in the area was isolated, and the germplasm screened against isolates of this race.

MATERIALS AND METHODS

Experimental Design

To represent the extensive germplasm available for organic farmers, seed companies, seed banks, and plant nurseries from the study area were interviewed. This resulted in the collection of a total of 32 genotypes belonging to different lettuce cultivar groups (Oak leaf, Butterhead, Batavia, and Cos) (**Table 1**). Landraces (16) and modern varieties (16) were represented equally in the study. Samples were grown during the winter season in two localities [Benifallet (40°58′22.46″N, 0°29′51.89″E) and La Múnia (41°19′26.8″N, 1°36′28.1″E)],

separated by 120 km. These localities were selected to represent different agroclimatic conditions relevant to lettuce production in Catalonia (**Figure 1**). Trials were conducted in fields that had been managed following organic farming practices for at least 15 years. Previous farmer management of the field trials consisted in a crop rotation based in many botanical families, including Brassicaceae, Liliaceae, and in less proportion Chenopioideae and Asteraceae during the fall season, and Liliaceae, Cucurbitaceae, and Solanaceae during the spring season. More specifically, the rotation previous to the transplant was broccoli (*Brassica oleracea* L. var *italica*) – cucumber (*Cucumis sativus* L.) in Benifallet, and broccoli-aubergine (*Solanum melongena* L.) in La Múnia. Both localities have similar edaphic and irrigation water characteristics, with slightly basic soil and water, clay loam soils, and low organic matter content (2.3% Benifallet, 1.2% La Múnia), but differ in the content of several macronutrients (N, P, K, and

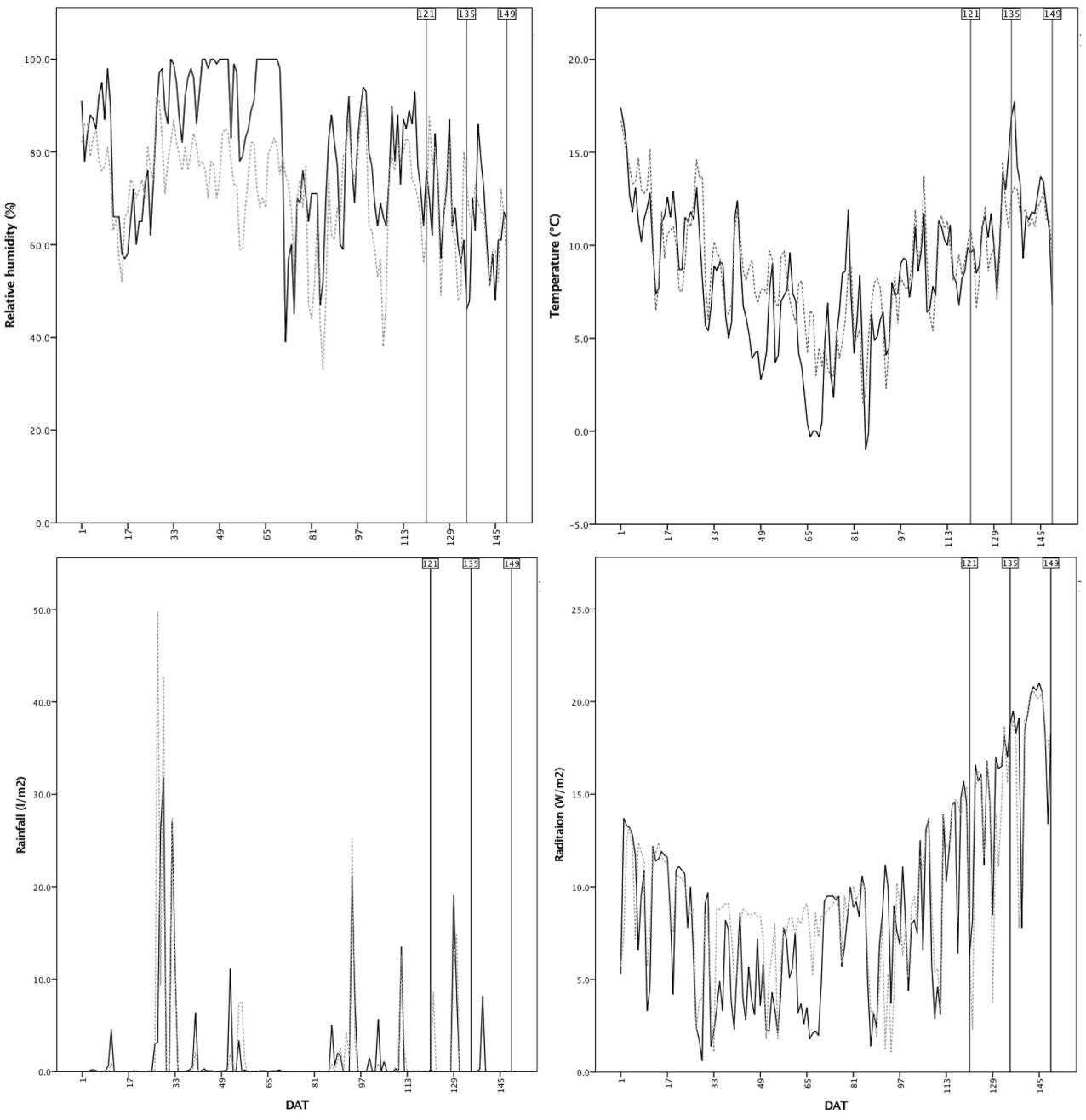

FIGURE 1 | Climatic conditions registered in the assays (Benifallet: continuous line; La Múnia: dashed line) during the cropping season: mean daily values for relative humidity, temperature, rainfall, and radiation. Harvesting dates are indicated with vertical lines. DAT, days after transplant.

Mg among others) (**Table 2**). Plants were irrigated with drip tapes (La Múnia) or sprinklers (Benifallet) and fertilized with a single application of cow manure prior to planting (equivalent of N 100 kg/ha). No phytosanitary treatments were applied during cultivation, and weeds were controlled manually. In each locality, a randomized block design was applied, with three replicates and 27 plants per plot, using a plant density of 6.67 plants/m^2. The total crop cycle length was 149 days (transplantation: 26/10/2016, late harvest: 23/03/2017).

Morphological Descriptors

Accessions were visually classified in the different cultivar groups using the classification proposed by the International Union for the Protection of New Varieties and Plants (International Union for the Protection of New Varieties of Plants [UPOV], 2016; **Table 1**). A total of 29 morphological descriptors were recorded for each accession, assessing different parts of the plant: cotyledons (color, anthocyanin presence, and shape), young leaf (position, color, anthocyanin distribution and intensity of coloration, blade border, and shape (outline, apex, base, and margin), vertical margin, undulation, and venation), adult outer leaf (color, anthocyanin distribution, and intensity, glossiness on the upper side, surface profile, blade border and shape (outline, apex, base, and margin), depth of incisions, blistering), head (head formation, shape in vertical section, overlapping of leaves), flower, and inflorescence, as proposed by Kristkova et al. (2008).

Agronomic Characterization

For each accession and locality, earliness was visually evaluated, and measured as the number of days between the transplant and the moment when 50% of plants reached the commercial stage [expressed as the number of days after transplant (DAT)]. According to these results, early-, mid-, and late-harvests were conducted at 121, 135, and 149 days after transplantation, in order to measure yield related traits during the length of the harvesting period, following the standard practices of farmers. In each harvest, 12 randomly selected individuals per accession (four from each block) were weighed. Both total weight (in g) and commercial weight (i.e., after external, old, and damaged leaves were removed according to typical farming practices; % of the total weight) were obtained. Incidence of *B. lactucae* was assessed on a per-plant basis at each harvest date using the following scale: 0 (no symptoms), 1 (few, small lesions), 2 (less than half of leaves with lesions), and 3 (high incidence, sporulating profusely), as proposed previously (Gustafsson, 1989).

Color and Chemical Evaluation

At mid-harvest, from the locality of La Múnia, three lettuces per accession were sampled and immediately processed for chemical and color analyses. Color (expressed as L^* (luminosity), a^* (ranging from green [negative values] to red [positive values]), b^* (ranging from blue [negative values] to yellow [positive values]) coordinates of the CIELAB color space), and chlorophyll content [measured as the index of absorbance difference (I_{AD})] of each accession were measured in the equatorial and terminal parts of three randomly selected inner leaves. A Konica Minolta CR-410 (Minolta, Osaka, Japan) and a DA-meter (TR-Turoni, Forli, Italy) were used for these analyses, respectively, with means of the three measurements used as the definitive result.

For chemical analyses, outer old leaves and the lettuce core were removed, with the remaining leaves washed in cold, running tap water. These samples were cut into pieces of approx. 2 cm^2 using a sharp stainless steel knife. Dry matter content was measured by drying the samples in an air oven (65°C, 72 h) and then weighing. For sugar analysis, 50 g of cut lettuce sample and 15 g of deionized water were mixed and homogenized in a blender. The addition of water was necessary to achieve a homogeneous sample. Sugars were extracted using deionized water. Approx. 30 g of homogenate was mixed with 20-30 mL of water, shaken for 15 min, and centrifuged. This was repeated three consecutive times and the three filtrated supernatants collated to give a volume of 100 mL of extract. Glucose, fructose, and sucrose were analyzed by HPLC, equipped with a pump (Beckman 110B, San Ramon, CA, United States), an

TABLE 2 | Physical and chemical characteristics of soil and irrigation water in La Múnia and Benifallet field trials.

	Soil			Irrigation water		
	Benifallet	**La Múnia**	**Units**	**Benifallet**	**La Múnia**	**Units**
pH	8.2	8		7.5	8.4	
Electrical conductivity	0.367	0.336	dS/m	0.962	1.38	dS/m
Organic matter	2.34	1.24	%			
Ca	43.1	31.72	%$CaCO_3$	7.26	4.89	meq/l
N-NO_3	24	49	mg/kg	0.05	1.02	meq/l
P $_{(Olsen)}$	33	151	mg/kg	4.61	<40	meq/l
K	428	205	mg/kg	0.05	<0.03	meq/l
Mg	252	378	mg/kg	3.58	7.5	meq/l
Ca	6422	4875	mg/kg	7.26	4.89	meq/l
Na	35	70	mg/kg	0.82	1.76	meq/l
Fe	2	0.56	mg/kg	<1	<25	meq/l
Mn	1.5	2.21	mg/kg	0.13	<0.1	meq/l
Textural class	Clay loam	Clay loam				

injector (Hewlett Packard Serie 1100, Walbrom, Germany) and a Refractive Index Detector (Beckman 156, United States). A Luna NH2 column, 250 mm × 4.6 mm (Phenomenex, Torrance, CA, United States) was used. Results are expressed as total sugars [mg/g fresh weight (fw)].

Screening for Resistance to *Bremia lactucae*

A lettuce from the La Múnia field showing a high incidence of *B. lactucae* (sporulating profusely) was harvested and brought to the laboratory. Conidiophores were extracted from the affected plant, and the isolate reproduced in the susceptible Green Towers variety. Once abundant new sporulations had been reproduced in these plants, these were used for characterization of the Catalonian *B. lactucae* isolate. Fifteen differential genotypes, defined by the International Bremia Evaluation Board (IBEB,[1] verified 25 June 2018), were used to help characterize the isolate. Inoculum was prepared for plant screening by shaking cotyledons bearing 3- to 4-day-old conidiophores with conidia in sterile distilled water. Seeds of screened lettuce varieties were sown in 40 cm × 30 cm × 10 cm trays filled with saturated substrate (30% white peat, 70% black peat; Neuhaus Huminsubstrat N3, Lassmann-Dellmann). Seedlings with fully expanded cotyledons (approx. 9-10 days after sowing) were inoculated by a sprayer with a suspension of 2×10^5 conidia/mL. Twenty plantlets of each variety were inoculated in three replicated experiments. After inoculation, the trays were covered with transparent plastic bags to create 100% humidity. Incubation was performed in a growth chamber under standard conditions, with a light intensity of 4000-5000 lux, continuous temperature of 16°C, and a 12-h photoperiod. The seedlings were observed at 7, 10, and 15 days after inoculation. Each plant variety was then scored for necrosis or asexual sporulation produced by *B. lactucae*. In the case of sporulation, four levels were established: 0 (absence of sporulation), 1 (weak sporulation, sporulation less than susceptible control), 2 (sparse sporulation), and 3 (sporulation comparable to the susceptible control). An accession was considered positive (exhibiting susceptibility to infection) when at least 5% of the tested plants gave a level of sporulation more than 2.

Finally, by using the same methodology as described above, we screened the experimental germplasm (**Table 1**) against the *B. lactucae* previously isolated. With this aim 160 plants per accession, divided in six replicates, were inoculated and the susceptibility to *B. lactucae* assessed. Results are expressed as the % of susceptible plants in each accession. In these experiments, we included Olaf variety as a susceptible control.

Farmer and Consumer Evaluations

With the aim of incorporating farmers in the characterization of the accessions, a farmer evaluation was organized in the field of La Múnia with the participation of 22 farmers. Participants evaluated visually the experimental plots, without any information regarding the name of the variety nor the origin (blind evaluation) and scored the accessions for the traits "commercial value" in a scale ranging from 0 (not interesting accession) to 10 (highly interesting accession) and "resistance to *B. lactucae*" in a scale ranging from 0 (non-resistant accession, high incidence) to 10 (resistant accession, without symptoms). In parallel, a consumer survey (untrained panellists) was organized in the sensory laboratory of the Barcelona School of Agricultural Engineering, with the participation of 47 consumers (55% female, 45% male; 45% between 19 and 34 years, 35% between 35 and 55 years, 20% between 56 and 70 years). Solely regular consumers of lettuce (at least one time per week) were selected, regardless of whether they were regular consumers of organic products (15% of participants). Each panelist received a whole lettuce to evaluate appearance and a cut sample to evaluate taste. Out of the 32 accessions, 16 (eight landraces and eight modern varieties) were rated on a 10 cm semi-structured scale from 0 ("Dislike") to 10 ("Extremely like") for "external appearance" and "taste acceptance" traits. Accessions were distributed randomly in two tasting sessions, in each of which half of the materials were evaluated. Samples were coded with a random three-digit number. Panellists did not receive any information regarding the objective of the study, neither about the origin of the varieties. Tasting sessions were carried out in a room designed for sensory analyses (International Organization for Standardization [ISO], 2017), using white light for the "external appearance" test and green light to mask the color during the "taste acceptance" test.

Statistical Analyses

Yield data (total weight and commercial weight) was analyzed within each locality and at each harvesting date by means of analysis of variance (ANOVA), using a full factorial model. We performed two independent ANOVA with the objective to assess (i) differences between cultivar groups [factors: accession (cultivar group), cultivar group, and block] and (ii) differences between origins (landrace or modern variety) within each cultivar group [factors: accession (origin), origin, and block]. Harvesting date and locality factors were not considered in the model, in order to obtain a more detailed description of the agronomic behavior of the accessions in each locality.

Resistance to *B. lactucae*, both in laboratory and field tests, and evaluations performed by farmers and consumers were analyzed by means of ANOVA considering solely the accession factor. For farmer and consumer data, each individual score was considerate as a replicate for the analysis. For significant factors, mean separation was conducted using the Student-Newman-Keuls test (*snk*, $p < 0.05$). A hierarchical cluster analysis (HCA), with average linkage applied as the grouping method, was carried out using Pearson distances for quantitative traits (chlorophyll, color, total sugar content, and dry matter) and Jaccard's distances for qualitative variables (morphological descriptors). Results were presented using a dendrogram by means of the same software. Principal component analysis (PCA) and Pearson bivariate correlation analyses were used to assess the variables underlying consumer and farmers preferences. SPSS (v.12.0, SPSS Inc., Chicago, IL, United States), Acuity (v.4.9, Axon Instruments, Union City, CA, United States), and R (R core team 2017; Agricolae, PCAmethods, and Ellipse packages) statistical

[1] http://www.worldseed.org/our-work/plant-health/other-initiatives/ibeb/

programs were used for univariate (ANOVA, mean separation), cluster, and PCA analyses, respectively.

RESULTS

Classification of Landraces According to Morphological Descriptors

Out of the 32 accessions studied, 11 belonged to Oak leaf, seven to Batavia and Cos, six to Butterhead, and one to Lollo cultivar groups (**Table 1**). The Amboise cultivar was initially included in the assay due to its classification in the Batavia group (according to the seed company description), but it was further reclassified as a Lollo cultivar. In each cultivar group both modern varieties and landraces were represented, except in the case of the Cos group, were solely landraces were identified. This was due to the particular interest of organic farmers in the *enciam negre* ("black lettuce") landrace, and the lack of available organic seeds of commercial cultivars in this group. The traditional names of landraces were highly diverse and did not offer appropriate information regarding the cultivar group pertinence. Such names referred to the crop cycle [e.g., *D'hivern FMA/252* ("winter lettuce," Cos); *De primavera FMA/87* ("spring lettuce," Butterhead)], origin [e.g., *Francès SIG/219/855* ("french lettuce," Oak leaf)] or specific morphological traits such as leaf type [e.g., *Cua d'oreneta* ("swallow tail," Oak leaf)], and color [e.g., *Enciam negre* ("black lettuce," five accessions, all belonging to the Cos type)]. In some cases, the same traditional name corresponded to multiple distinct cultivar groups, for instance, the *Carxofet* ("little artichoke") accessions, two of which were classified as Batavia and one as Butterhead.

The groups obtained by means of HCA on the 29 morphological descriptors studied (**Figure 2**) were highly consistent with the cultivar group pertinence in the case of Batavia, Butterhead, and Oak leaf. Batavia and Butterhead were the most homogeneous cultivar groups, with all of the accessions

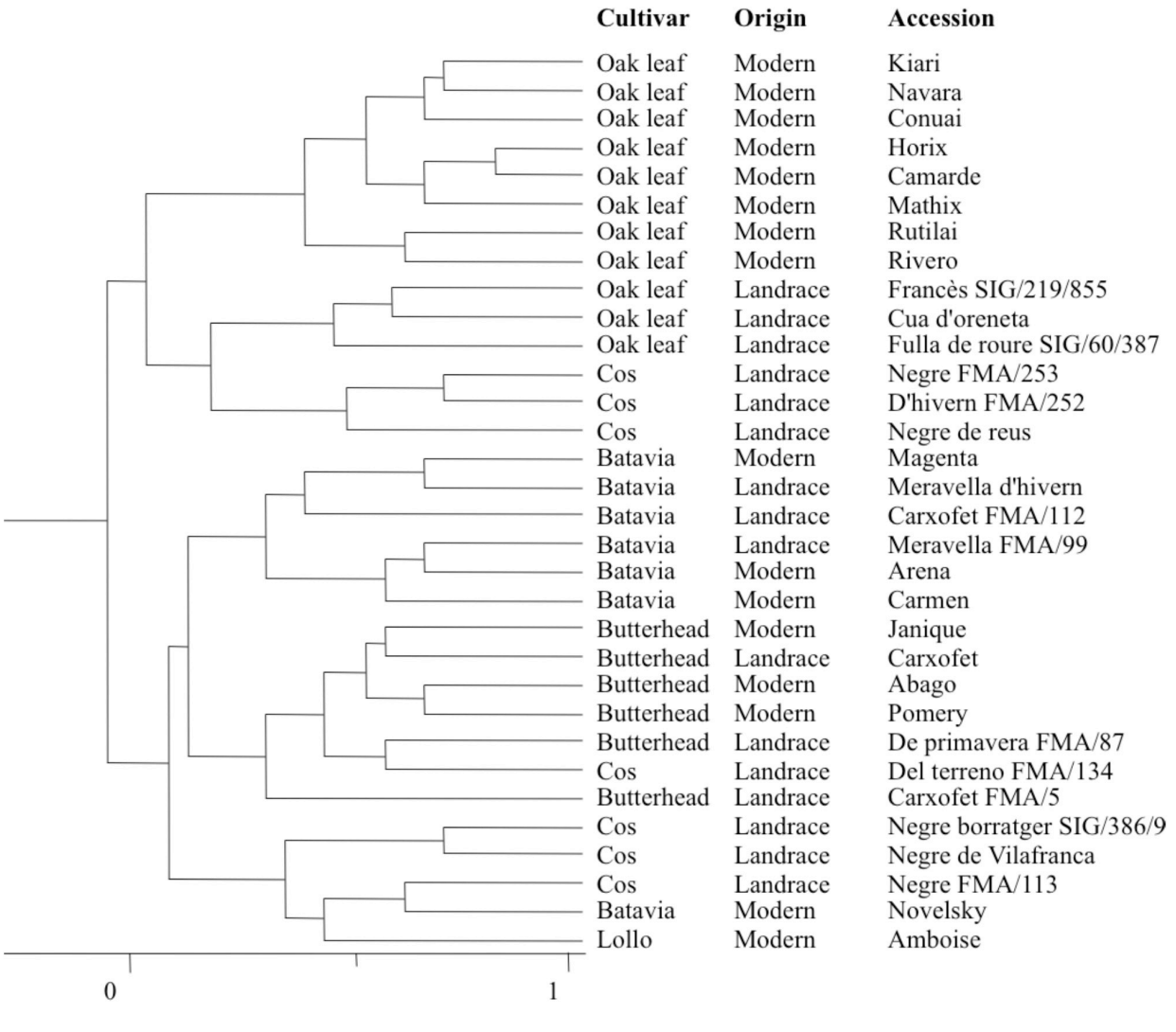

FIGURE 2 | Relativeness between accessions according to the hierarchical cluster analysis performed with 29 morphological descriptors.

belonging to each group clustering together in the HCA [with the exception of Novelsky - this Batavia type was more related to Amboise (Lollo) and *Negre FMA/113* ("black lettuce," Cos)]. Within the Oak leaf group, two clusters were identified, clearly separating landraces from modern varieties. Cos seemed a highly divergent group, forming two distinct clusters [one more related to Oak leaf landraces, and the other to Amboise (Lollo)]. Finally, one Cos accession [*Del terreno FMA/134* ("field lettuce")] clustered together with the Butterhead group.

Agronomic Characterization

Earliness ranged from 107 to 140 DAT in La Múnia and from 122 to 150 DAT in Benifallet, with Butterhead cultivar group showing the highest earliness in both localities (significantly different to the other groups at $p < 0.05$, except with Oak leaf) (**Table 1**). According to these results we decided to perform 3 harvests (early-harvest, 121 DAT; mid-harvest, 135 DAT; late-harvest: 149 DAT) with the objective to assess the yield of each accession during the length of the harvesting period. Results for total weight (g) and commercial weight (%) revealed that major differences were related to cultivar groups rather than to the genetic origin (landrace vs. modern) of the accessions (**Table 3** and **Supplementary Table S1**). In both localities, and regardless of the harvesting date, the higher values for total weight were obtained by Cos and Butterhead accessions. No general pattern for the modern/landrace comparison was found. For example, landraces yielded significantly higher total weights in the Oak leaf cultivar group, while in the case of Butterhead, agronomic

TABLE 3 | Comparisons between cultivar groups, and between genetic origins (landraces vs. modern varieties) within cultivar groups, for the agronomic traits studied [total weight (g) and commercial weight (%)] in *Lactuca sativa* L. accessions grown in (a) La Múnia, and (b) Benifallet.

	Early-harvest				Mid-harvest				Late-harvest			
	Total weight (g)		Commercial weight (%)		Total weight (g)		Commercial weight (%)		Total weight (g)		Commercial weight (%)	
(a) La Múnia												
Cultivar groups												
Batavia	299.5	c	78.5	b	398.4	c	77.6	ns	617.4	c	86.2	a
Butterhead	359.6	b	81.7	b	480.8	b	78.8	ns	640.3	b	82.8	b
Cos	477.8	a	78.5	b	567.0	a	75.9	ns	783.8	a	81.3	b
Oak leaf	244.8	d	84.4	a	332.9	d	81.6	ns	441.8	d	83.0	b
Origin												
Batavia												
Modern	304.2	*ns*	79.3	*ns*	397.5	*ns*	78.4	*ns*	592.8	ns	85.1	ns
Landrace	293.5		77.8		399.9		76.5		649.4		87.7	
Butterhead												
Modern	327.6	*	84.9	**	452.0	*ns*	81.1	*ns*	602.6	*	85.8	*
Landrace	391.6		78.5		509.6		76.5		678.0		79.8	
Oak leaf												
Modern	203.2	***	85.7	ns	280.0	***	83.8	*	371.3	***	82.8	*ns*
Landrace	358.8		80.9		473.9		76.9		619.3		83.5	
(b) Benifallet												
Cultivar groups												
Batavia	180.8	b	79.2	a	445.8	b	89.2	a	578.1	c	92.2	a
Butterhead	369.6	a	82.2	a	779.9	a	79.4	c	742.6	b	78.9	c
Cos	376.7	a	73.7	b	789.3	a	77.5	c	893.6	a	78.2	c
Oak leaf	193.3	b	78.3	a	512.1	b	81.6	b	537.2	c	85.5	b
Origin												
Batavia												
Modern	188.5	*ns*	77.9	*ns*	456.9	*ns*	89.4	*ns*	571.1	*ns*	92.0	ns
Landrace	170.6		80.9		431.2		88.9		588.4		92.5	
Butterhead												
Modern	417.8	***	92.2	**	790.8	***	81.9	**	935.4	*	81.9	*
Landrace	321.4		72.4		680.7		76.5		604.9		76.5	
Oak leaf												
Modern	165.9	***	80.2	***	437.5	***	84.0	***	410.7	***	86.4	*ns*
Landrace	266.2		73.5		711.0		75.3		874.4		83.2	

*Data were collected at early, mid, and late-harvests (121, 135, and 149 days, respectively). Within columns, letters indicate significant differences between corresponding cultivar groups (Student-Newman-Keuls test, at $p < 0.05$), and ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns (not significant) indicate significant differences between landrace and modern genotypes within each cultivar group.*

behavior was highly dependent on the locality (with higher landrace yields in Benifallet, and lower in La Múnia), signaling an important G × E effect. No significant differences were found between landraces and modern varieties in the Batavia group.

Commercial weight (%) was more dependent on the harvesting date and also showed a clearer separation between traditional and modern varieties. In all of the cases studied where significant differences were detected, higher commercial weights were recorded in the modern accessions. However, it should be noted that commercial weight was higher than 70% (i.e., 30% of the total weight should be discarded prior to commercialization) for all accessions, and even for accessions with severe reduction of the total weight [e.g., Arena (69.8%) or *Negre borratger SIG/386/935* "black lettuce" (72.4%)], harvested lettuces reached the minimum standards for commercialization.

Chemical and Color Evaluation

Analogously with the results from the morphological characterization (**Figure 2**), HCA performed on color and chemical composition revealed a consistent clustering of the cultivar groups (**Figure 3**). The principal factor of classification (groups A-D vs. group E) was found to be related to the chemical composition (sugar content, dry matter, and chlorophyll) and to intensity of red color (a^* coordinate) measured in the terminal part of the leaf. Cos and Oak leaf landraces, and one traditional Butterhead accession, clustered together (group E), and were characterized by high levels of sugars, dry matter, chlorophyll content and yellow color (b^* coordinate, measured in the terminal part of the leaf), and low levels of red color (a^* coordinate, terminal). Batavia (group A) and Butterhead (group B) accessions showed some relativeness in comparison with the rest of the collection, being characterized by low levels of sugars, dry matter, and chlorophyll content. Nevertheless, the two cultivar groups were distinct with respect to their color traits: luminosity (L^* coordinate, both equatorial, and terminal) and yellow color (b^* coordinate, equatorial) were higher in Butterhead accessions, while red color (a^* coordinate, terminal) was higher in Batavia accessions. Most of the Oak leaf modern varieties clustered together (group C), characterized by their color profile in the equatorial part of the leaf [high values for red color (a^*) and low values for luminosity (L^*) and yellow (b^*)], but with a similar chemical profile to Butterhead and Batavia groups. Thus, a clear separation between Oak leaf modern varieties and landraces was observed, with landraces characterized by higher sugar, dry matter, and chlorophyll content, and modern varieties showing a more intense red color (a^*) in the equatorial and terminal part of the leaves. Finally, a more heterogeneous group (group D), formed by modern varieties of Oak leaf (Mathix, Kiari), Batavia (Arena), and Lollo (Amboise) cultivar groups, showed a similar profile to the Oak leaf group (group C), but with some differences related to the color at the terminal part of the leaves.

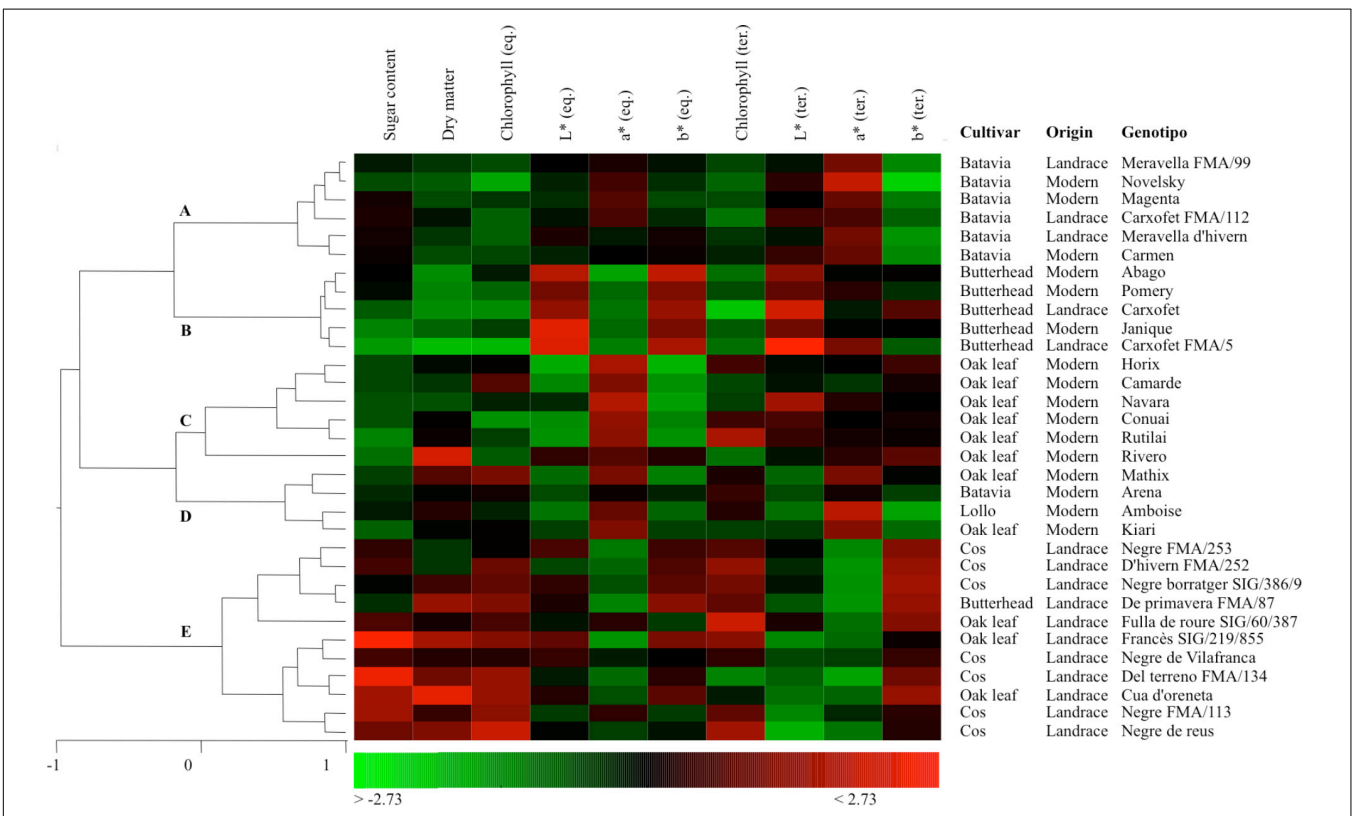

FIGURE 3 | Hierarchical cluster analysis from chemical and color traits. Values are represented as a heatmap according to the scale below. eq., measured in the equatorial part of the leaf; ter., measured in the terminal part of the leaf.

TABLE 4 | Susceptibility of *Lactuca sativa* L. accessions to the *Bremia lactucae* pathogen, as scored in laboratory and field studies.

				Laboratory test		Field test (0–3)				
Variety	**Accession**	**Origin**	**Cultivar group**	**Susceptible plants (%)**		**Benifallet**		**LaMunia**		**Resistance (qualitative)**
1	Conuai	Modern	Oak leaf	0	f	0.1	j	0.5	hij	R-R-R
2	Rutilai	Modern	Oak leaf	0	f	0.0	j	0.5	hij	R-R-R
3	Abago	Modern	Butterhead	0	f	0.1	j	0.2	j	R-R-R
4	Novelsky	Modern	Batavia	0	f	1.3	gh	1.5	abcdefg	R-IR-IR
8	Pomery	Modern	Butterhead	0	f	0.1	j	0.2	j	R-R-R
9	Camarde	Modern	Oak leaf	0	f	0.0	j	0.5	hij	R-R-R
10	Amboise	Modern	Lollo	0	f	0.2	j	0.2	j	R-R-R
13	Janique	Modern	Butterhead	0	f	0.4	ij	0.3	j	R-R-R
15	Mathix	Modern	Oak leaf	0	f	1.7	fg	0.4	ij	R-S-R
24	De primavera FMA/87	Landrace	Butterhead	0	f	2.0	def	0.8	ghij	R-S-IR
16	Horix	Modern	Oak leaf	6	f	0.1	j	0.2	j	R-R-R
12	Kiari	Modern	Oak leaf	16	f	0.1	j	0.4	ij	R-R-R
23	D'hivern FMA/252	Landrace	Cos	39	e	2.6	abc	1.5	abcdefg	S-S-S
31	Carxofet	Landrace	Butterhead	47	de	2.6	abcd	1.2	cdefgh	S-S-IR
7	Carmen	Modern	Batavia	52	cde	0.7	hij	1.2	cdefg	S-IR-IR
18	Negre borratger SIG/386/935	Landrace	Cos	65	bcd	2.9	a	2.1	a	S-S-S
30	Negre de Vilafranca	Landrace	Cos	65	bcd	2.8	ab	2.1	ab	S-S-S
19	Francès SIG/219/855	Landrace	Oak leaf	67	bcd	2.0	cdef	1.6	abcdef	S-S-S
20	Negre FMA/113	Landrace	Cos	67	bcd	2.8	ab	1.8	abcd	S-S-S
22	Carxofet FMA/112	Landrace	Batavia	67	bcd	0.9	hi	1.1	defgh	S-IR-IR
29	Negre de reus	Landrace	Cos	67	bcd	2.5	abcde	1.5	abcdefg	S-S-S
17	Fulla de roure SIG/60/387	Landrace	Oak leaf	70	bcd	2.6	abc	1.7	abcde	S-S-S
28	Meravella d'hivern	Landrace	Batavia	72	bcd	1.0	hi	1.3	cdefg	S-IR-IR
21	Negre FMA/253	Landrace	Cos	73	bcd	2.9	a	1.8	abcd	S-S-S
11	Magenta	Modern	Batavia	77	abc	1.2	gh	1.2	cdefgh	S-IR-IR
25	Meravella FMA/99	Landrace	Batavia	77	abc	0.7	hij	1.3	bcdefg	S-IR-IR
32	Cua d'oreneta	Landrace	Oak leaf	79	abc	1.9	ef	1.0	efghi	S-S-IR
14	Navara	Modern	Oak leaf	82	ab	2.7	abc	1.2	cdefgh	S-S-IR
6	Arena	Modern	Batavia	89	ab	0.7	hij	1.6	abcdef	S-IR-S
5	Rivero	Modern	Oak leaf	89	ab	2.8	ab	1.9	abc	S-S-S
Olaf	Olaf	Control		100	a					S–
26	Del terreno FMA/134	Landrace	Cos			2.2	bcdef	0.9	fghij	-S-IR
27	Carxofet FMA/5	Landrace	Butterhead			2.6	abc	1.4	abcdefg	-S-IR

The table includes variety identification number, accession name, origin (modern or landrace), cultivar group, and susceptibility to infection in laboratory testing (% of susceptible plants, six replicates), and in Benifallet and La Múnia field studies (graded 0-3, 26 replicates). Field study grading was carried out as follows: 0 (no symptoms), 1 (few, small lesions), 2 (less than half of leaves with lesions), and 3 (high incidence, sporulating profusely). Final laboratory-Benifallet-La Múnia susceptibility scores are reported as R, resistant; IR, intermediate resistance; S, susceptible. Within columns, letters indicate significant differences between accessions (Student-Newman-Keuls test, at $p < 0.05$).

Resistance to *Bremia lactucae*

The Catalonian isolate of *B. lactucae* showed no correspondence to any of the races previously reported by the IBEB, indicating that a previously undescribed race is present in the fields of this area. Regarding the susceptibility of the experimental germplasm, significant differences between accessions were detected, both in tests performed in the laboratory and in the field. All of the landraces, except *De primavera FMA/87* ("spring lettuce," Butterhead type), were susceptible to the *B. lactucae* race isolated from the area (**Table 4**). Moreover, five of the 16 modern varieties studied were also susceptible. Results obtained in the laboratory were significantly correlated ($p < 0.001$) with field observations carried out by researchers in both localities [Benifallet ($r = 0.62$) and La Múnia ($r = 0.79$)] (**Table 5**), although in some cases, slightly different responses between laboratory and field tests were identified. In most of such cases, accessions characterized as susceptible in the laboratory were classified as intermediately resistant in the field. The higher correlation between the laboratory and La Múnia tests is consistent with the fact that the *B. lactucae* race used in the laboratory test was isolated from this particular field.

Farmer and Consumer Preferences

An ANOVA performed on farmer and consumer evaluations revealed significant differences between accessions for all of the traits under study ($p < 0.05$), and differences between origins

TABLE 5 | Pearson bivariate correlations between agronomic, chemical, and color traits, together with farmer and consumer evaluations.

	Commercial weight (%)	Susceptibility to B. lactucae (Laboratory test, %)	Susceptibility to B. lactucae (Benifallet field, %)	Susceptibility to B. lactucae (La Múnia field, %)	Total sugars (mg/ g fw)	Dry Matter (%)	Commercial value (farmer)	Resistance (farmer)	Appearance (consumer)	Taste acceptance (consumer)	Chlorophyll (eq.)	L* (eq.)	a* (eq.)	b* (eq.)	Chlorophyll (ter.)	L* (ter.)	a* (ter.)	b* (ter.)
Total weight (g)		0.*	0.62***	0.51**	0.57***			−0.6***	−0.5*			0.43 *	−0.66***	0.51**			−0.51**	
Commercial weight (%)		−0.49**	−0.53**	−0.70***				0.49**										
Susceptibility to *B. lactucae* (Laboratory test, %)			0.62***	0.79***	0.42*			−0.70***										
Susceptibility to *B. lactucae* (Benifallet field, %)				0.78***	0.35*			−0.83***					−0.35*				−0.5**	0.53**
Susceptibility to *B. lactucae* (La Múnia field, %)								−0.81***										
Total sugars (mg/g fw)						0.49**		−0.37*			0.65***		−0.38*			−0.60***	−0.52**	
Dry matter (%)											0.65***				0.40*	−0.77***	−0.41*	0.45*
Commercial value (farmer)								0.50**	0.56*									
Resistance (farmer)																		
Appearance (consumer)													0.59*	−0.60*				
Taste acceptance (consumer)																		
Chlorophyll (eq.)															0.55**	−0.77***	−0.67***	0.57***
*c** (eq.)													−0.78***	0.87***	−0.39*	0.46**		
*a** (eq.)														−0.94***			0.49**	
*b** (eq.)																		
Chlorophyll (ter.)																−0.52**	−0.44*	0.38*
*c** (ter.)																		
*a** (ter.)																		−0.90***
*b** (ter.)																		

*Solely significant correlations are shown. ***, **, and * indicate levels of significance as $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively.*

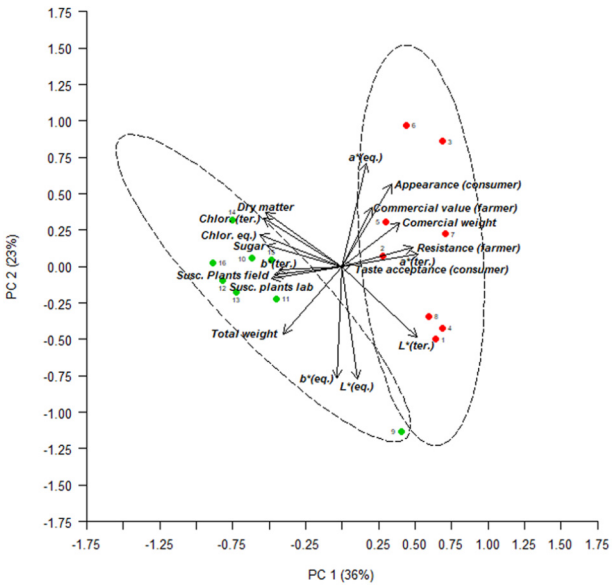

FIGURE 4 | Plot from the two first principal components (59% of the total variation) in the PCA estimated from all the data of the experiment (agronomic, chemical, color, and resistance to *B. lactucae*, plus consumer and farmer evaluations), considering the 16 accessions evaluated by consumers (codes are presented in **Table 1**). Green points, landraces; red points, modern varieties.

(landraces vs. modern varieties) for the traits "resistance to *B. lactucae*" (field evaluation made by farmers; modern varieties yielding higher scores) and "external appearance" (laboratory evaluation made by consumers; modern varieties being higher scored).

To introduce farmer and consumer evaluations, a multivariate analysis was conducted with all of the data from the experiment recorded in the field of La Múnia (except for morphological descriptors). The first two components of the PCA, which accounted for 59% of the total variation, were plotted (**Figure 4**). PC1 (36% of the total variation), which was positively correlated to commercial weight, and negatively to susceptibility to *B. lactucae* (both for laboratory and field tests), provided a clear distinction between landraces and modern varieties. Moreover, farmers' evaluations regarding the commercial value and resistance to *B. lactucae* variables, and consumers' ratings (regarding external appearance) showed a clear tendency to prefer modern varieties, being sensitive to plants with intact leaves and negatively influenced by total weight trait. Some varieties such as Mathix (Oak leaf), Conuai (Oak leaf), or Novelsky (Batavia) seem to fit with farmer and consumer preferences. Consumer evaluations on taste acceptance were not discriminant between accessions nor origins, according to the PCA analysis.

For a greater understanding of the phenotypic traits underlying farmer and consumer preferences, a Pearson correlation analysis was conducted, considering all of the traits evaluated (**Table 5**). Farmer evaluations regarding the resistance to *B. lactucae* were highly correlated to the susceptibility tests performed in the laboratory ($r = -0.70$) and in the field (La Múnia $r = -0.83$; Benifallet $r = -0.81$), signaling their strong ability to discriminate between accessions regarding this trait. The sign of the correlation (negative) is due to the different scales of evaluation used by researchers (susceptibility) and farmers (resistance). Resistance measured by farmers was also correlated with total weight ($r = -0.60$), commercial weight ($r = 0.49$), and with their perception of the commercial value of each accession ($r = 0.50$), signaling that this group of characteristics drive farmers' preferences for lettuce cultivars. With regard to consumer evaluations, few significant correlations were obtained. External appearance correlated positively with commercial value scored by farmers ($r = 0.56$) and with red color (a^*, equatorial, $r = 0.59$), and negatively with yellow color (b^*, equatorial, $r = -0.60$).

Regarding relationships between agronomic, chemical, and color traits, most of the correlations were detected between the color coordinates L^*, a^*, and b^* (equatorial/terminal). Total sugars were correlated with chlorophyll content measured at the equatorial part of the leaf ($r = 0.65$), but not when measured at the terminal part. Moreover, sugars were also related to the color of the leaves, showing significant and negative correlations with red color (a^*) measured at the equatorial ($r = -0.38$) and terminal ($r = -0.52$) positions, and with luminosity (L^*) at the terminal position only ($r = -0.60$). Finally, chlorophyll content (equatorial and terminal) was also related to L^*, a^*, and b^* color coordinates, when evaluated in the terminal part of the leaf.

DISCUSSION

In comparison with other major horticultural crops, genetic and phenotypic profiles of lettuce landraces have been scarcely studied in the scientific literature. Landrace varieties of crops are rapidly regaining importance in the commercial field, promoted mainly by the interest of specific niche markets, such as organic food production. Organic farmers are therefore interested in identifying lettuce landrace varieties (i.e., pure lines) that show promising agronomic performance, while also presenting distinctive organoleptic and nutritional quality traits. Our study shows that, when comparing landraces with modern varieties, the major source of variation is the cultivar group rather than the origin of the material. Moreover, in our study, we characterized the *B. lactucae* race present in the experimental field of La Múnia, which showed no correspondence with any of the previously reported races (Parra et al., 2016). Landraces were highly susceptible to this race, both when assessed in the field and in the laboratory (using inoculated plants), as solely one of the 16 landraces evaluated [*De primavera FMA/87* ("spring lettuce," Butterhead type)] showed resistance to this pathogen. This accession can be considered a "traditionalized" modern variety (i.e., a modern variety that has been multiplied by farmers and recently adopted as a traditional variety), although this remains unclear. By contrast, most of the modern varieties showed good levels of resistance (with only five out of 16 exhibiting susceptibility), signaling that the genetic resistance conferred by *Dm* genes, already introgressed in modern cultivars, is functional

against this new race. Susceptibility to *B. lactucae* is the major drawback currently limiting the cultivation of landraces by farmers (van Treuren et al., 2013). Therefore, breeding programs directed at introducing genetic resistance to landraces is a priority with the objective of recovering the cultivation of these varieties. Prior to undertaking these breeding programs, the composition and distribution of Catalonian *B. lactucae* isolates should be analyzed, and later decide which genes should be strategically introduced into the improved landraces. Nevertheless, despite the higher incidence of *B. lactucae* in landraces, all of the landraces studied reached commercially acceptable standards in this experiment. For some landraces, commercial weight reached only 70% of the total weight, but this was compensated by a higher total biomass production.

With respect to the quality traits compared in this study (total sugars, dry matter, and chlorophyll content), the higher scores were identified in landraces. Some accessions such as *Francès SIG/219/855* ("french lettuce," total sugars: 15.6 mg/g fw) or *Del terreno FMA/134* ("field lettuce," 15.2 mg/g fw), among others, showed promising values regarding sugar content when compared with the remaining accessions of the experiment (range of variation: 5.2-12.9 mg/g fw) and with results obtained by other authors (Still, 2007; Ouzounidou et al., 2013; López et al., 2014). Nevertheless, sugar content and the other quality traits are known to demonstrate significant seasonal (Suthumchai et al., 2007) and year-to-year fluctuations (Mampholo et al., 2016), so further studies should assess G × E interactions and the optimal harvesting time for each landrace. Moreover information about the differences between landraces and modern varieties regarding other important quality traits such as nitrate content, carotenoid antioxidants and other compounds will be of great interest to boost the revaluation of these varieties.

Multivariate analyses, conducted on morphological descriptors (**Figure 2**) and chemical and color traits (**Figure 3**), revealed a consistent grouping for the Butterhead and Batavia accessions, and for the modern varieties of Oak leaf. By contrast, Oak leaf landraces were highly distinct to their modern counterparts, and Cos landraces showed a higher within-variety diversity. In the case of morphological descriptors, these included several traits not directly related to the external appearance of the mature lettuce (e.g., traits measured on seedling, young leaf, or stem), so these results can offer further clues regarding the phylogenetic relationships of each cultivar group. Cos lettuces have been described as one of the most ancient cultivated varieties (de Vries, 1997), and it has been hypothesized that the other cultivar groups have been derived from this source of variability (Mou, 2008). Our results show that Cos lettuces present a high intra-varietal diversity, which is in accordance with previous results obtained using molecular markers (Sharma et al., 2017).

Lettuce is a highly heterogeneous plant, which complicates methodological protocols to analyze quality traits. For instance, some correlations with chemical composition were significant solely when color or chlorophyll were measured in the terminal or equatorial part of the leaves (total sugars, chlorophyll, and color). Correlation between total sugars and chlorophyll content seem very interesting for breeders, as chlorophyll content has also been positively correlated with beta-carotene and lutein concentrations (Mou, 2005). Therefore, with farmers (and then breeders) initially selecting for green colored lettuces, they have in fact been selecting indirectly for increased sugar and carotenoid content. Nevertheless, the differences in composition between cultivar groups are very high (Mou, 2005; Simonne et al., 2001), and some correlations may be provoked by the differences between cultivar groups rather than because of pleiotropic effects. Thus, further research should focus on dissecting the genetic basis of these traits.

Considering that landraces are gaining interest in specific markets characterized by an emphasis on local production, organic farming and consumer demand for natural foods (Brush, 2000), we suggest that research programs intended to recover landraces should incorporate farmers and consumers in their phenotyping platforms. In our study farmers showed a high capacity to qualitatively characterize the genetic diversity related to the agronomic behavior. Moreover, consumers and farmers seem influenced by similar traits when scoring the varieties, being positively influenced by commercial weight (i.e., how intact the leaves of a variety appear), and negatively influenced by total weight and susceptibility to *B. lactucae*. Consumer agreement with farmer evaluations is particularly important, as it represents the potential to design an ideotype fulfilling the needs from both groups. Regarding lettuce color, it seems that consumers are particularly receptive to lettuces with intense red color on the internal part. By contrast, less interesting results were obtained when assessing the taste acceptance by consumers, probably due to the existence of different consumer segments, as reported for other crops (Causse et al., 2010), and their lower experience in characterizing materials.

CONCLUSION

In agreement with previous analyses, this study identified the high intra-varietal diversity within the Cos cultivar group and characterized the principal differences with Butterhead, Batavia, and Oak leaf types. It showed that when comparing landraces with modern varieties, the principal factor of variance was related to the cultivar group. However, the higher scores for total sugars, dry matter, or chlorophyll content identified in landraces signals that these varieties show extremely promising characteristics. Regarding the agronomic behavior, yield, and resistance to the *B. lactucae* race isolated in the area were characterized in the germplasm collection, identifying one landrace that showed a high level of resistance. Finally, farmers showed a high technical capacity for characterizing the genetic diversity. It is therefore proposed that farmers and consumers should be included in the phenotyping platforms in future research projects aiming for the recovery of lettuce landraces.

AUTHOR CONTRIBUTIONS

JM made substantial contributions to the conception or design of the work; coordinated the field trials and phenotyping activities;

participated in the analysis and interpretation of data; drafted the manuscript; and gave final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. AR conducted the tasting sessions; participated in the analysis and interpretation of data; and revised the article critically, and final approval of the version to be published. BC made substantial contributions to the conception or design of the work; performed the agronomic characterization; organized the farmer evaluation; and interpreted the data. MF, CC, and SS made substantial contributions to the conception or design of the work; performed the laboratory tests for resistance to *B. lactucae*; and gave final approval of the version to be published. JS revised the article critically and gave final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This work was funded by the Ministry of Agriculture, Livestock, Fisheries and Food of the Generalitat de Catalunya through the contract ARP/374/2015.

ACKNOWLEDGMENTS

The authors acknowledge farmers and consumers who participated in the experiment, and the students Albert Jo and Paula Parellada for their assistance in the phenotyping of the materials.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01491/full#supplementary-material

REFERENCES

Brush, S. B. (2000). *Genes in the Field. On-Farm Conservation of Crop Diversity*. Boca Raton, FL: IDRC.

Casals, J., Casañas, F., and Simó, J. (2017). Is it still necessary to continue to collect crop genetic resources in the Mediterranean area? A case study in Catalonia. *Econ. Bot.* 71, 330–341. doi: 10.1007/s12231-017-9392-0

Casañas, F., Simó, J., Casals, J., and Prohens, J. (2017). Toward an evolved concept of landrace. *Front. Plant Sci.* 08:145. doi: 10.3389/fpls.2017.00145

Causse, M., Friguet, C., Coiret, C., LéPicier, M., Navez, B., Lee, M., et al. (2010). Consumer preferences for fresh tomato at the European scale: a common segmentation on taste and firmness. *J. Food Sci.* 75, S531–S541. doi: 10.1111/j.1750-3841.2010.01841.x

de Vries, I. M. (1997). Origin and domestication of Lactuca sativa L. *Genet. Resour. Crop Evol.* 44, 165–174. doi: 10.1023/A:1008611200727

Food and Agriculture Organization Corporate Statistical Database [FAOSTAT] (2016). *Food and Agricultural Commodities Statistics*. Available at: http://faostat.fao.org/

Gepts, P. (2006). Plant genetic resources conservation and utilization. *Crop Sci.* 46, 2278–2292. doi: 10.2135/cropsci2006.03.0169gas

Gustafsson, I. (1989). Potential sources of resistance to lettuce downy mildew (*Bremia lactucae*) in different *Lactuca* species. *Euphytica* 40, 227–232. doi: 10.1007/BF00024516

Hammer, K., and Teklu, Y. (2008). Plant genetic resources: selected issues from genetic erosion to genetic engineering. *J. Agric. Rural Dev. Trop. Subtrop.* 109, 15–50.

International Organization for Standardization [ISO] (2017). *Sensory Analysis - General Guidance for the Design of Test Rooms (ISO 8589:2007)*. Genova: ISO.

International Union for the Protection of New Varieties of Plants [UPOV] (2016). *Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability - Lettuce*. Genova: UPOV. doi: 10.1007/s00122-005-0162-5

Jansen, J., Verbakel, H., Peleman, J., and van Hintum, T. J. L. (2006). A note on the measurement of genetic diversity within genebank accessions of lettuce (*Lactuca sativa* L.) using AFLP markers. *Theor. Appl. Genet.* 112, 554–561. doi: 10.1007/s00122-005-0162-5

Kristkova, U. E., Dolezaolva, I., Lebeda, A., Vinter, V., and Novotná, A. (2008). Description of morphological characters of lettuce (*Lactuca sativa* L.) genetic resources. *Hortic. Sci.* 35, 113–129. doi: 10.1016/j.jfca.2013.10.001

López, A., Javier, G.-A., Fenoll, J., Hellín, P., and Flores, P. (2014). Chemical composition and antioxidant capacity of lettuce: comparative study of regular-sized (Romaine) and baby-sized (little gem and mini Romaine) types. *J. Food Compos. Anal.* 33, 39–48. doi: 10.1016/j.jfca.2013.10.001

Mampholo, B. M., Maboko, M. M., Soundy, P., and Sivakumar, D. (2016). Phytochemicals and overall quality of leafy lettuce (*Lactuca sativa* L.) varieties grown in closed hydroponic system. *J. Food Qual.* 39, 805–815. doi: 10.1111/jfq.12234

Michelmore, R., and Wong, J. (2008). Classical and molecular genetics of *Bremia lactucae*, cause of lettuce downy mildew. *Eur. J. Plant Pathol.* 122:19. doi: 10.1007/s10658-008-9305-2

Morris, M. L., and Bellon, M. R. (2004). Participatory plant breeding research: opportunities and challenges for the international crop improvement system. *Euphytica* 136, 21–35. doi: 10.1023/B:EUPH.0000019509.37769.b1

Mou, B. (2005). Genetic variation of beta-carotene and lutein contents in lettuce. *J. Am. Soc. Hortic. Sci.* 130, 870–876.

Mou, B. (2008). "Lettuce," in *Handbook of Plant Breeding, Vegetables I, Asteraceae, Brassicaceae, Chenopodicaceae, and Cucurbitaceae*, eds J. Prohens and F. Nuez (New York, NY: Springer), 75–116. doi: 10.1023/A:1025933613279

Negri, V. (2003). Landraces in central Italy: where and why they are conserved and perspectives for their on-farm conservation. *Genet. Resour. Crop Evol.* 50, 871–885. doi: 10.1023/A:1025933613279

Ouzounidou, G., Paschalidis, C., Petropoulos, D., Koriki, A., Zamanidis, P., and Petridis, A. (2013). Interaction of soil moisture and excess of boron and nitrogen on lettuce growth and quality. *Hortic. Sci. Repub.* 40, 119–125. doi: 10.1007/s10681-016-1687-1

Parra, L., Maisonneuve, B., Lebeda, A., Schut, J., Christopoulou, M., Jeuken, M., et al. (2016). Rationalization of genes for resistance to *Bremia lactucae* in lettuce. *Euphytica* 210, 309–326. doi: 10.1007/s10681-016-1687-1

Prada, D. (2009). Molecular population genetics and agronomic alleles in seed banks: searching for a needle in a haystack? *J. Exp. Bot.* 60, 2541–2552. doi: 10.1093/Jxb/Erp130

Sharma, S., Kumar, P., Gambhir, G., Kumar, R., and Srivastava, D. K. (2017). Assessment of genetic diversity in lettuce (*Lactuca sativa* L.) germplasm using RAPD markers. *3 Biotech* 8:9. doi: 10.1007/s13205-017-1039-4

Simonne, E., Simonne, A., and Wells, L. (2001). Nitrogen source affects crunchiness, but not lettuce yield. *J. Plant Nutr.* 24, 743–751. doi: 10.1081/PLN-100103667

Still, D. W. (2007). "Lettuce," in *Genome Mapping and Molecular Breeding in Plants*, ed. C. Kole (Berlin: Springer Heidelberg), 127–140. doi: 10.3923/jbs.2007.752.758

Suthumchai, W., Matsui, T., Kawada, K., Kosugi, Y., and Fujimura, K. (2007). Seasonal fluctuations of sucrose metabolizing enzymes activities and sugar contents in lettuce (*Lactuca sativa* L.). *J. Biol. Sci.* 7, 752–758. doi: 10.1007/s10681-012-0765-2

Tesfaye, L. M., Bink, M. C. A. M., van der Lans, I. A., Gremmen, B., and van Trijp, H. C. M. (2013). Bringing the voice of consumers into plant breeding with Bayesian modelling. *Euphytica* 189, 365–378. doi: 10.1007/s10681-012-0765-2

van de Wouw, M., Kik, C., van Hintum, T., van Treuren, R., and Visser, B. (2010). Genetic erosion in crops: concept, research results and challenges. *Plant Genet. Resour. Util.* 8, 1–15. doi: 10.1017/s1479262109990062

van Treuren, R., van der Arend, A. J. M., and Schut, J. W. (2013). Distribution of downy mildew (*Bremia lactucae* regel) resistances in a genebank collection of lettuce and its wild relatives. *Plant Genet. Resour.* 11, 15–25. doi: 10.1017/S1479262111000761

Veteläinen, M., and Maxted, N. (2009). *European Landraces: On-Farm Conservation, Management and Use*. Rome: Bioversity International. doi: 10.17660/ActaHortic.2008.782.4

Vicente, M. J., Conesa, E., Franco, J. A., Esteva, J., and Martínez-Sánchez, J. J. (2008). Genetic variability in lettuce (*Lactuca sativa*) germplasm using morphologic and molecular analyses. *Acta Hortic.* 782, 59–66. doi: 10.17660/ActaHortic.2008.782.4

Villa, T. C. C., Maxted, N., Scholten, M., and Ford-Lloyd, B. (2005). Defining and identifying crop landraces. *Plant Genet. Resour.* 3, 373–384. doi: 10.1079/PGR200591

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 March 2018
doi: 10.3389/fpls.2018.00378

Faba Greens, Globe Artichoke's Offshoots, Crenate Broomrape and Summer Squash Greens: Unconventional Vegetables of Puglia (Southern Italy) With Good Quality Traits

Massimiliano Renna[1], Angelo Signore[1], Vito M. Paradiso[2] and Pietro Santamaria[1]*

[1] Department of Agricultural and Environmental Science, University of Bari Aldo Moro, Bari, Italy, [2] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Marco Leonti, Università degli Studi di Cagliari, Italy
Sofia Plexida, University of Thessaly, Greece
Inacio de Barros, Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Brazil
Milton Kanashiro, Embrapa Amazonia Oriental (Embrapa Easter Amazon), Brazil

***Correspondence:**
Angelo Signore
angelo.signore@uniba.it

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Plant Science

Received: *20 December 2017*
Accepted: *07 March 2018*
Published: *27 March 2018*

Citation:
Renna M, Signore A, Paradiso VM and Santamaria P (2018) Faba Greens, Globe Artichoke's Offshoots, Crenate Broomrape and Summer Squash Greens: Unconventional Vegetables of Puglia (Southern Italy) With Good Quality Traits. Front. Plant Sci. 9:378. doi: 10.3389/fpls.2018.00378

Globe artichoke (*Cynara cardunculus* L. subsp. [L.] *scolymus* Hayek), summer squash (*Cucurbita pepo* L.) and faba bean (*Vicia faba* L.) are widely cultivated for their immature inflorescences, fruits and seeds, respectively. Nevertheless, in some areas of Puglia (Southern Italy), other organs of these species are traditionally used as vegetables, instead of being considered as by-products. Offshoots (so-called *cardoni* or *carducci*) of globe artichoke, produced during the vegetative growing cycle and removed by common cultural procedures, are used like to the cultivated cardoons (*C. cardunculus* L. var. *altilis* DC). The stems, petioles, flowers and smaller leaves of summer squash are used as greens (so-called *cime di zucchini*), like other leafy vegetables such as chicory (*Cichorium intybus* L.) and Swiss chard (*Beta vulgaris* L.). Also the plant apex of faba bean, about 5–10 cm long, obtained from the green pruning, are used as greens (so-called *cime di fava*) like spinach leaves. Moreover, crenate broomrape (*Orobanche crenata* Forssk.), a root parasite plant that produces devastating effects on many crops (mostly legumes), is used like asparagus (*Asparagus officinalis* L.) to prepare several traditional dishes. In this study ethnobotanical surveys and quality assessment of these unconventional vegetables were performed. For their content of fiber, offshoots of globe artichokes can be considered a useful food to bowel. Summer squash greens could be recommended as a vegetable to use especially in the case of hypoglycemic diets considering both content and composition of their carbohydrates. For their low content of nitrate, faba greens could be recommended as a substitute of nitrate-rich leafy vegetables. Crenate broomrape shows a high antioxidant activity and may be considered as a very nutritious agri-food product. Overall, the results of the present study indicate that offshoots of globe artichoke, summer squash greens, faba greens and crenate broomrape have good potential as novel foods, being nutritious and refined products. Their exploitation aiming to the obtainment of labeled and/or new potential ready-to-eat retail products could satisfy the demand for local functional foods.

Keywords: by-products, culinary use, ethnobotany, landrace, nutritional value, traditional agri-food products

INTRODUCTION

Puglia region (Southern Italy), placed in the center of the Mediterranean basin, has a long tradition in vegetable crops. It is very rich in landraces obtained by farmers themselves through repeated simple selection procedures generation after generation (Elia and Santamaria, 2013). Thus, many of these landraces are listed as an item in the 'List of Traditional Agri-Food Products' of the Italian Ministry of Agricultural, Food and Forestry Policies, since their processing, preservation and aging methods are consolidated in time, harmonious, according to traditional rules, for a period not less than 25 years. It is interesting to highlight that in Puglia region a relevant number of vegetable-based traditional dishes may be found. Therefore, several landraces of vegetables, for which there is a strong link with the regional traditions, are used as ingredients for preparing several dishes of the Puglia's cuisine (Renna et al., 2015a).

The landrace vegetables of Puglia are appreciated both as refined food and for the intake of several healthy nutrients. For example, fruits of *Carosello* and *Barattiere* (herbaceous plants belonging to *Cucumis melo* L. species) are consumed at the immature stage, instead of cucumbers, not only for their organoleptic traits but also for the high potassium content and low amount of sodium and sugars (Serio et al., 2005). Polignano Carrot (a multicolored landrace of *Daucus carota* L., locally so-called *Carota di Polignano*) is greatly appreciated by people for its special taste, texture, flavor, fragrance and great variety of colors, that range from yellow to dark purple in the outer core and from pale yellow to light green in the inner core (Cefola et al., 2012; Renna et al., 2014b). Moreover, for its high antioxidant activity as well as for its high content in total phenols, carotenoids and β-carotene it can be regarded also as a functional food (Cefola et al., 2012; Renna et al., 2014b). *Galatina* and *Molfettese* stem chicories (two landraces of *Cichorium intybus* L., Catalogna group), useded both raw and cooked, represent a refined and nutritious vegetables, because of the presence of several healthy compounds as well as their low nitrate content (Renna et al., 2014a; Testone et al., 2016; D'Acunzo et al., 2017).

An interesting and time-honored custom of Puglia counts the culinary use of several plant parts, which can be considered as "unconventional" agri-food products. This because for a few vegetable species some plant parts are usually treated as by-products or wastes of the agri-food chain and not as a food. For example, globe artichoke (*Cynara cardunculus* L. subsp. [L.] *scolymus* Hayek) is widely cultivated for its immature inflorescences which can be considered a very important food product of the Mediterranean diet. Plants of globe artichoke are generally propagated vegetatively by offshoots (**Figure 1A**) which are continuously produced during the vegetative cycle. Thus, a part of them is removed from the field by common cultural procedures. These offshoots (**Figure 1A**) are usually considered as by-products, but in some areas of Puglia (Southern Italy) they are traditionally used as a food ingredient, like to the cultivated cardoons (*C. cardunculus* L. var. *altilis* DC). Summer squash (*Cucurbita pepo* L.) is widely cultivated in the world for both its fruits and flowers. Nevertheless, in some areas of Puglia region its stems, petioles and smaller leaves are used as "greens" (**Figure 1B**), like other leafy vegetables such as chicory (*Cichorium intybus* L.) and Swiss chard (*Beta vulgaris* L.). The faba bean (*Vicia faba* L.) is widely cultivated in the world for its seed, especially as a dry legume. In some areas of Puglia the plant apex of faba bean, about 5–10 cm long, obtained from the green pruning, are used as "greens" (**Figure 1C**) like spinach leaves (*Spinacia oleracea* L.). Finally, crenate broomrape (*Orobanche crenata* Forssk.), a parasite plant (**Figure 1D**) that produces devastating effects on many crop (mostly legumes). In a few areas of Puglia this parasite is considered a wild edible plant used, like asparagus (*Asparagus officinalis* L.), for preparing several traditional dishes (Renna et al., 2015a,b).

The culinary use of these products in Puglia, like several wild edible plants, has ancient origins and is due to the food scarcity and poverty of the ancestors in the past. Thus, instead of being eliminated as by-products, the culinary use of these plant parts enabled farmers to gain a precious extra food source (Bianco et al., 1998; Elia and Santamaria, 2013).

Nowadays these unconventional vegetables could satisfy the needs of specific markets. This because niche food products, founded on quality and agricultural biodiversity and intended for local use, seems to meet requirements of consumers about safety and genuineness. Therefore, it is of high importance to evaluate the nutritional traits of these products for further exploitation activities aimed to produce high added value products and by reinforcing local rural economies. Moreover, especially for specific niche markets, characterized by a high demand of local products grown through sustainable techniques, it is essential to disseminate the knowledge about landraces (Signore et al., 2014). At the same time, also local traditions and cultural identity of people could be preserved and promoted, assuming that each product is intimately linked to the local identity, with specific geographic, climatic, environmental and cultural characteristics (Renna, 2015).

To the best of our knowledge, there is a lack of information in literature with regard to ethnobotanical information as well as to the nutritional characterization of these unconventional vegetables obtained from Puglia landraces. Starting from these considerations, a compositional analysis of globe artichoke's offshoots, faba greens, summer squash greens and crenate broomrape was performed. The general goal was to assess some quality traits, such as proteins, lipids, total carbohydrates, fiber, mineral content and antioxidant activity, in order to furnish an overview and evaluate the potential of these foodstuffs as traditional agri-food products for culinary uses.

MATERIALS AND METHODS

Study Site and Ethnobotanical Surveys

Puglia region (**Figure 2**), 350 km long and 60 km narrow, is largely open to the Adriatic and Ionian seas with a coastal zone of nearly 800 km. Its area of about 19,360 km^2 shows more than 60% of territory below 200 m above sea level, with some peaks of more than 1000 m located in the North-East and North-West. The climate is semi-arid Mediterranean, characterized by high temperatures and drought in summer and

FIGURE 1 | Offshoots removed from plants of globe artichoke **(A)**. Summer squash greens **(B)**. Faba greens **(C)**. Crenate broomrape **(D)**.

moderately cold temperatures and rainfall in winter. Thanks to its geographical length and variety of orographic and pedoclimatic conditions, Puglia region shows an interesting richness of vegetables crop.

Ethnobotanical studies on landraces cultivated by seed savers were conducted though field studies and *ad hoc* investigations about the culinary customs and folk use of vegetable landrace. Additionally, ethnobotanical papers as well as folkloric and gastronomic literature, were also considered.

Plant Material and Sample Preparation

Four different types of unconventional vegetables were collected and analyzed: globe artichoke's offshoots, faba greens, summer squash greens and crenate broomrape. Offshoots were removed for plants of two different globe artichoke landrace: *Carciofo di Lucera* (late genotype) and *Locale di Mola* (early genotype) (**Figure 2**). Summer squash greens and faba greens were collected from plants of landraces of Andria and Mola di Bari, respectively, (**Figure 2**). Crenate broomrape was collected from yield cultivated with different landraces of faba bean. Immediately after harvesting, samples of each type of unconventional vegetable were refrigerated and then transferred to the laboratory to be processed and analyzed. After removing inedible parts, samples were washed with tap water, blotted dry with paper towels and cut to obtain edible portions of vegetables. Each replicate was freeze-dried (ScanVac CoolSafe 55-9 Pro; LaboGene ApS, Lynge, Denmark) and then packed in hermetic jars and stored in the dark at $-21 \pm 1°C$ until the analyses were carried out.

Chemical Analysis

Proximate analysis of samples was carried out as follows: ashes were determined by muffle furnace according to AOAC method 923.03 (AOAC, 1991); proteins content ($N \times 6.25$) was determined by Kjeldahl nitrogen according to AOAC method 955.04 (AOAC, 1991); fat content was determined by Soxhlet extraction according to AOAC method 920.39 (AOAC, 1991); dietary fiber content was determined by enzymatic-gravimetric procedure according to AOAC method 991.43 (AOAC, 1991); moisture content by automatic moisture analyzer (Mod. MAC 110/NP, Radwag Wagi Elektroniczne, Radom, Poland) at 105°C; and total carbohydrates were calculated by difference of protein, lipid and ash on the dry matter basis.

Before inulin extraction, globe artichoke's offshoots were subdivided into root, external leaves and edible parts. Inulin was extracted from globe artichoke's offshoots according to Ronkart et al. (2007) with some adaptations. 250 grams of minced sample were extracted with 800 mL of hot water (80°C) for 90 min. The pH of water was adjusted to 6.8 with NaOH in order to avoid inulin hydrolysis at pH < 6. Extracted juice was filtered on a Büchner funnel with 11 μm Whatman filters N. 1. Inulin was then precipitated by two cycles of freezing/thawing followed by

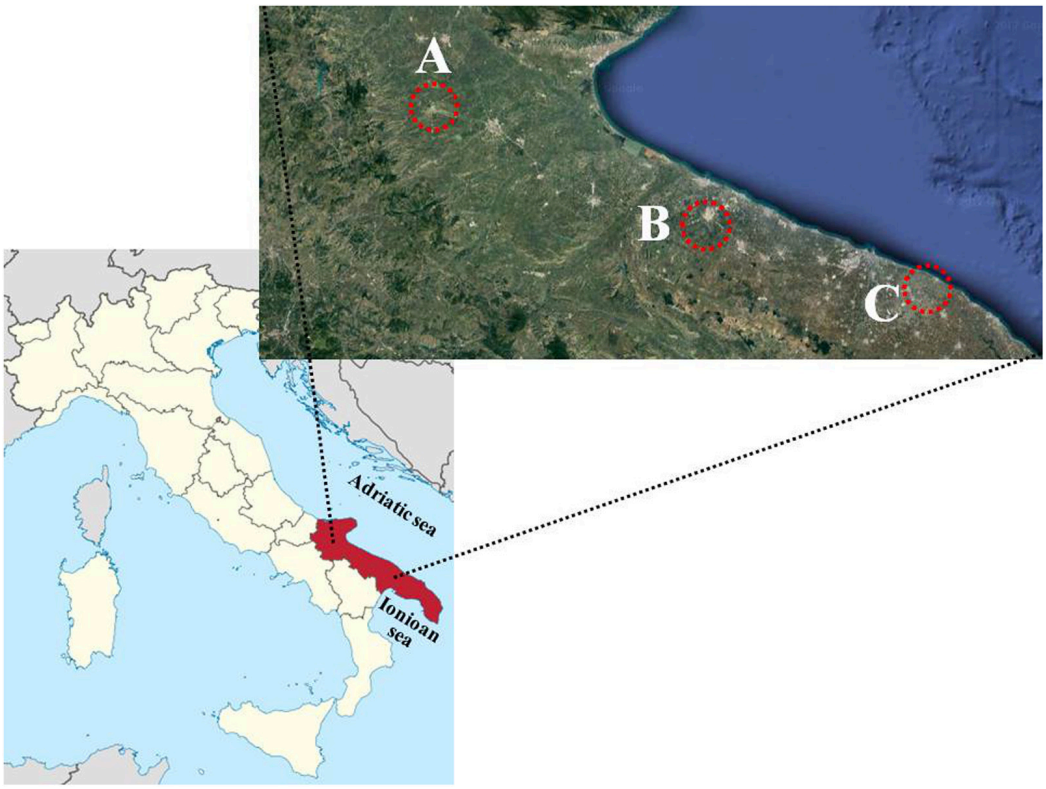

FIGURE 2 | Map of Italy with Puglia Region in red and the areas (within the circle) where offshoots of globe artichokes and summer squash greens were grown: Lucera **(A)**; Andria **(B)**; Mola di Bari **(C)**.

centrifugation at 7500 × *g* and 10°C for 15 min. The pellet was washed with 10 mL of acetone, centrifuged, dried and weighted.

Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical scavenging capacity test, according to Difonzo et al. (2017). Samples (0.1 g) were extracted with 5 mL water:methanol (80:20) for 2 h in tubes covered with aluminum foil. Extracts were then centrifuged for 15 min at 15000 *g* and 24°C. The supernatant was recovered and filtered with PTFE septa (0.45 μm). Extracts (50 μL) were added to 950 μL of 0.08 mM DPPH in ethanol. The mixture was shaken and left at room temperature in the dark for 30 min. The decrease of the absorbance at 517 nm was measured using a Cary 60 Agilent spectrophotometer (Agilent Technologies, Milan, Italy). The results were expressed in μmol Trolox equivalents (TE) g^{-1} dry weight. Each sample was analyzed in triplicate.

For inorganic ion content an ion exchange chromatography (Dionex DX120; Dionex Corporation, Sunnyvale, CA, United States) with a conductivity detector was performed as reported by D'Imperio et al. (2016). Nitrate (NO_3^-) content was determined in 0.5 g dried sample using an IonPac AG14 precolumn and an IonPac AS14 separation column (Dionex Corporation). Cation contents (Na^+, K^+, Mg^{2+}, and Ca^{2+}), were determined in 1 g dried sample using an IonPac CG12A guard column and an IonPac CS12A analytical column (Dionex Corporation). In order to determine glucose, fructose and saccharose contents, samples were prepared by protocols used by Renna et al. (2014a) and analyzed by ion chromatography (Dionex DX-500; Dionex Corp., Sunnyvale, CA, United States) using a pulsed amperometric detector. Peak separation was performed using a Dionex CarboPac PA1 and isocratic elution with 50 mmol L^{-1} NaOH.

Experimental Design and Data Analysis

Three series for each unconventional vegetable were harvested in order to provide independent replicates. For each replicate, all samples were then mixed well in order to obtain a bulk sample. Chemical analyses were performed in triplicate for each bulk sample. To detect statistical significance, ANOVA was applied (GLM procedure, SAS software) and means were separated by the Student–Newman–Keuls (SNK) test.

RESULTS

Ethnobotanical Information

During the first half of the 20th century, some authors (Gramignani, 1934; Tamaro, 1937; Turchi, 1957) have described the techniques to be applied to artichoke plants for producing tender and light colored offshoots, so-called "*gobbi*" (hunchbacked) because they were curved to the soil. In Italian language the offshoots of globe artichoke are named *cardoni* or *carducci*. It is likely that these local names are due to

the similarity of the globe artichoke's offshoots to both cultivated and wild cardoons. It is also interesting to note that in Puglia about 300 families with the name "*Cardone*" can be found, while in Italy over 1,300 families with this name can be found (Renna and Santamaria, 2017). Before being used as a food ingredients, fibrous parts of the offshoots must be eliminated and, after boiling, a soaking process in water for some hours is need for reducing their bitter taste (Renna et al., 2015a). In 2016, the offshoots of globe artichoke have been listed as an item in the 'List of Traditional Agri-Food Product of Puglia' of the Italian Ministry of Agricultural, Food and Forestry Policies, because of their consolidated culinary use in the region for over 25 years. Indeed, in Puglia it is well known the home-made culinary preparation of this unconventional vegetable. Moreover, some gastronomic books of traditional cuisine reported several recipes, such as first courses, side dishes, main courses and pizza with using the offshoots of globe artichokes (**Figure 3**) similar to recipes based on cardoons and heads of globe artichoke (Sada, 1991).

Summer squash greens are traditionally used as a food almost exclusively in some territories of the Southern Italy, especially in Puglia. In Italian summer squash greens are named *cime di zucchina*. Most likely, the culinary use of this vegetable originated from the peasantry cuisine of these territories. Effectively, stems, petioles and smaller leaves of the summer squash are one of the main ingredients of the so-called "survival cuisine", which in periods of poverty has constrained to exploit any edible part of the vegetable plants (Renna and Santamaria, 2017). Therefore, toward the end of the productive cycle, our ancestor harvested stems, petioles and smaller leaves in order to better exploit all parts of the summer squash plant. Moreover, in the past, farmers used as summer squash greens also the excess seedlings, so-called *siverchi*, that were removed from the field as a consequence of overabundant use of seeds during sowing respect to the optimal plant density in the field (Renna and Santamaria, 2017). The first known information regarding the culinary use of summer squash greens is found in an ancient book dating back to 1576 (Ingrassia, 1576). In this book, the author has described the food use of the summer squash greens between some good nutrition practices useful for preventing the plague contagion that in those times was much feared. Moreover, summer squash greens were also described as a food ingredient in another ancient book dating back to 1824 (Soderini, 1824). Nowadays, summer squash greens are traditionally used especially with the pasta for preparing first courses but also for preparing side dishes like for other conventional leafy vegetables such as chicory and Swiss chard. Anyway, before to use as food ingredient it is important to remove all fibrous filaments from stems and petioles of the summer squash greens (**Figure 4**).

In Italian faba greens are named *cime di fava*. The green pruning of the faba plant apex is an ancient operation that farmers carried out to satisfy several cultural needs such as the deterrent action against aphids. It is not a case that this specific action is well described by Margaroli in an ancient book dating back to 1831 (Margaroli, 1831). According to folk knowledge, in the past the plant apexes removed by means of pruning was not considered as a by-product but as a springy vegetable. Indeed, faba greens can be eaten raw, for example in salads, or cooked like spinach to be used in pasta dishes or into quiches and omelets (**Figure 5**). It is likely that the similarity of the faba greens to spinach leaves (**Figure 1C**) was a decisive factor in using this part of the plant as a food. Nevertheless, taste, smell and texture of the faba greens are very different from ones of spinach leaves.

The earliest known information on crenate broomrape as a food is found in Pliny the Elder's Book of Natural History: *. . . it is a small leafless stem that can be eaten. . .* (Bianco et al., 2009). Similarly, the culinary use of crenate broomrape was described in an ancient book of the 15th century (Bianco et al., 2009). Nevertheless, several authors have been reported negative information describing this parasitic plant. For example, Matthioli (1613) and Redi (Bianco et al., 2009) called broomrape "wolf herb" on account of its ability to "eat up" adjacent plants (Bianco et al., 2009). In some areas of Puglia the term s*porchia* is one of the common local names used for *O. crenata* L.. It is interesting to underline that the etymology of this local name, is due to the very high quantity of seeds produced by this parasitic plant and descends from the Latin "*exporculare*" (Bianco et al., 2009). Crenate broomrape was a real disaster for the main legume crops, therefore its elimination from the fields allowed to preserve an important crop and obtain an extra food product. It is not a case that its culinary use is reported in some books as well as in ethnobotanical and scientific papers (Bianco et al., 1998, 2009; Perrino et al., 2004; Renna et al., 2015a,b). In some areas of Puglia, crenate broomrape is widely used for several traditional dishes. Probably, its resemblance to large asparagus was a decisive factor in harvesting and consuming this parasitic plant. Stems of crenate broomrape are cleaned, washed and boiled. In a similar way for offshoots of globe artichoke, also for crenate broomrape a soaking process in water (12–24 h) is needed for reducing their bitter taste (**Figure 6**). Today. the culinary use of crenate broomrape is a part of the traditional Puglia's cuisine with a linkage to the Mediterranean Diet (Renna et al., 2015a). In 2015 crenate broomrape has been listed as an item in the 'List of Traditional Agri-Food Product of Puglia'. It is interesting to highlight that nowadays local farmers harvest and sell crenate broomrape for up to three times as much as fresh broad beans (Renna et al., 2015b).

Nutritional Traits

Table 1 shows the proximate composition of globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape. The highest water content was found in summer squash greens, followed by *Locale di Mola* and *Lucera* globe artichoke's offshoots, while the lowest content was found in both faba greens and crenate broomrape (about 85 g 100 g^{-1} fresh weight - FW - on average). As regard the protein content the highest value was found in faba greens. Summer squash greens showed a content of protein about 75% lower respect to faba greens and about 40% lower respect both crenate broomrape and offshoots of *Lucera* globe artichoke. At the same time, no significant differences about protein content were found between offshoots of *Locale di Mola* globe artichoke and summer squash greens (**Table 1**). Crenate broomrape showed the highest content of both total lipid and carbohydrate, while in summer squash greens was found the lowest content of these food components

FIGURE 3 | Dishes based on offshoots of globe artichoke: boiled *Locale di Mola* **(A)** and *Lucera* **(B)** landraces; baked potatoes, rice and offshoots **(C)**; pizza with tomato sauce, mozzarella cheese, olives, capers and offshoots **(D)**.

(**Table 1**). It is interesting to highlight that the carbohydrate content in crenate broomrape was higher of about 32, 78, 114, and 281% compared with faba greens, offshoots of *Lucera* globe artichoke, offshoots of *Locale di Mola* globe artichoke and summer squash greens, respectively (**Table 1**). With respect the dietary fiber, offshoots of *Lucera* globe artichoke showed a higher content respect both summer squash greens and crenate broomrape (**Table 1**). Moreover, no significant differences were found between faba greens and offshoots of *Locale di Mola* globe artichoke also as compared with all other unconventional vegetables. Finally, ashes content was highest in both offshoots of *Lucera* globe artichoke and summer squash greens, and lowest in faba greens, crenate broomrape and offshoots of *Locale di Mola* globe artichoke.

In globe artichoke's offshoots the mean value of the inulin content was of 1.04 g 100 g^{-1} FW and 1.41 g 100 g^{-1} FW, respectively, for *Lucera* and *Locale di Mola* landraces.

The content of principal cations and nitrate in globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape is reported in **Table 2**. The highest content of sodium was found in offshoots of *Locale di Mola* globe artichoke, while the lowest content was found in summer squash greens, faba greens and crenate broomrape (about 19.7 mg 100 g^{-1} FW on average). Offshoots of *Lucera* globe artichoke showed a sodium content about 70% lower compared with offshoots of *Locale di Mola* globe artichoke, but about 2.4-fold higher compared with summer squash greens, faba greens and crenate broomrape (**Table 2**). Potassium content was highest in both summer squash greens and offshoots of *Locale di Mola* globe artichoke (about 489 mg 100 g^{-1} FW on average), followed by faba greens and both crenate broomrape and offshoots of *Lucera* globe artichoke that showed the lowest values. As regards magnesium, summer squash greens showed a content about 54% higher compared with crenate broomrape, while no significant differences were found respect to other unconventional vegetables (**Table 2**). The highest content of calcium was found in both types of globe artichoke's offshoots and summer squash greens (about 99 mg 100 g^{-1} FW on average), while the lowest content was found in both faba greens and crenate broomrape (about 57 mg 100 g^{-1} FW on average). As regards nitrates, the lowest content was found in summer squash greens. Faba greens and offshoots of *Lucera* globe artichoke showed a nitrate content (about 97 mg 100 g^{-1} FW on average) about 7.4-fold higher compared with summer squash greens and about 90% higher compared with crenate broomrape, while no significant differences were found between faba greens and both type of globe artichoke's offshoots (**Table 2**).

Table 2 shows the content of glucose, fructose and sucrose in globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape. The highest glucose content was found in both types of globe artichoke's offshoots (about 791 mg 100 g^{-1} FW on average). In comparison to these unconventional

FIGURE 4 | Home-made culinary preparation of summer squash greens: removal of fibrous filaments **(A)**; cutting **(B)**; edible parts **(C)**; pasta with summer squash greens **(D)**.

vegetables, the glucose content in crenate broomrape was about 40% lower, while in both summer squash greens and faba greens the glucose content (147 mg 100 g^{-1} FW on average) was about 81% lower. Fructose content in crenate broomrape was about 3.2-fold higher respect to offshoots of *Locale di Mola* globe artichoke and about sevenfold higher compared with other unconventional vegetables. As regards the sucrose content, the highest value was found in faba greens, while no significant differences were found between other unconventional vegetables.

The antioxidant activity of globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape is reported in **Figure 7**, expressed in μmol Trolox equivalents (TE) 100 g^{-1} FW. Summer squash greens showed the lowest value followed by offshoots of globe artichokes. Instead, the highest value was found in samples of crenate broomrape.

DISCUSSION

Since offshoots of globe artichoke can be used in several traditional dishes like for recipes based on cardoons and heads of globe artichoke, it could be interesting to compare the proximate composition between these conventional and unconventional vegetables. Offshoots of both *Lucera* and *Locale di Mola* globe artichoke showed lower water content respect to cardoons but similar to heads of globe artichoke (**Tables 1**, **3**). With respect to the content of protein and carbohydrates, offshoots of globe artichoke showed lower content respect to heads of globe artichoke but higher respect to cardoons (**Tables 1**, **3**). The fiber content in offshoots of globe artichokes represents about 58 and 63% of total carbohydrate, respectively, for *Locale di Mola* and *Lucera* landraces (**Table 1**). It is also interesting to highlight that the AOAC Method 991.43 partially measures inulin and does not measure at all fructo-oligosaccharides (both prebiotic fiber). On the other hand, significant inulin contents were detected in offshoots. Therefore, actual total dietary fiber levels in offshoots are higher than those measured by this official method (Prosky and Hoebregs, 1999; Lattanzio et al., 2009). Thus, offshoots of globe artichokes may be considered a useful food to maintain bowel regularity in the short term, and to potentially afford a protection against chronic diseases in the long term. This because all the fibers of globe artichoke's offshoots as a whole (prebiotic and other ones) can contribute directly to fecal bulking and indirectly, by stimulating the increase of probiotic microbial biomass (Flamm et al., 2001). Regarding the content of main cations (**Table 2**) offshoots of globe artichoke may be considered as low contributors to the sodium supply especially for *Lucera* landrace. Effectively, 100 g of this landrace of globe artichoke's offshoots supplies about 3.2% of the Na daily intake (1.5 g per day) (European Food Safety Authority, 2006).

FIGURE 5 | Dishes based on faba greens: first course pasta-based **(A)**; boiled **(B)**; salad with cherry tomatoes and carrots **(C)**; baked omelet **(D)**.

As for *Locale di Mola* landrace, the same serving size of globe artichoke's offshoots supplies about 10.7% of the daily intake. The higher sodium content in offshoots of *Locale di Mola* globe artichoke is probably due to proximity of the cultivated fields to sea (**Figure 2**) and therefore to the use of brackish water coming from aquifers. Moreover, the higher sodium content may have affected the potassium content considering the lower K content in *Locale di Mola* offshoots respect *Lucera* ones as well as the Na/K inverse correlation (**Table 2**), since sodium can substitute the K in the plant osmotic regulation. Anyway, both types of globe artichoke's offshoots showed a higher sodium content respect to other unconventional vegetables (**Table 2**) confirming that plants belonging to the *Asteraceae* generally show a higher Na content in comparison with other botanic familes (Bianco et al., 1998). As for potassium, the results of the present study suggest that offshoots of *Locale di Mola* globe artichoke can be considered as low contributors to the daily supply of this cation. Considering an amount of 4.7 g K per day as an adequate intake (Food and Nutritional Board, 2005), 100 g of this unconventional vegetable could satisfy only about the 4% of this intake. Regarding the nitrate content (**Table 2**), offshoots of *Locale di Mola* globe artichoke may be considered as vegetables with a middle content (500–1000 mg kg^{-1} FW) like for cabbage (*Brassica oleracea* L. var. *capitata* [L.] DC.), dill (*Anethum graveolens* L.), radicchio (*Cichorium intybus* L., group *Rubifolium*), turnip (*Brassica rapa* L. subsp. *rapa* Thell.) and broccoli raab (*Brassica rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort.), while offshoots of *Lucera* globe artichoke may be considered as vegetables with a relatively high content (1000–2500 mg kg^{-1} FW) like for celeriac (*Apium graveolens* L. group *Rapaceum*), fennel (*Foeniculum vulgare* Mill. var. *azoricum*), endive (*Cichorium endivia* L., group *Crispum*) and leek (*Allium porrum* L.) (Santamaria, 2006). For both *Lucera* and *Locale di Mola* landrace, the nitrate content in the offshoots is higher with respect to the heads of globe artichoke, which may be considered as vegetables with a very low content (<200 mg kg^{-1} FW) of this anion. This because NO_3^- content differs in the various parts of a plant and its content in the stem (i.e., offshoots of globe artichoke) is higher respect to inflorescences (Santamaria, 2006). Regarding the sugar content, it is interesting to highlight that offshoots of globe artichoke show a similar total amount for both *Lucera* and *Locale di Mola* landrace as well as a similar composition of the different type of sugars. In fact, glucose represents the most abundant sugar, accounting for about 60 and 75% of the total amount, respectively, for *Lucera* and *Locale di Mola* landrace (**Table 2**). Therefore, these results suggest that the sugar content in offshoots of globe artichoke is scarcely influenced by genetic and environmental factors. The antioxidant activity measured in artichoke offshoots by DPPH assay was of about 1500 μmol TE 100 g^{-1} FW, showing comparable levels to those reported for other leafy vegetables by Pennington and Fisher (2009). The same authors assigned artichoke (inflorescence) to the vegetable class with antioxidant activity higher than 9000 μmol TE 100 g^{-1} FW. Differences in antioxidant activity between offshoots and

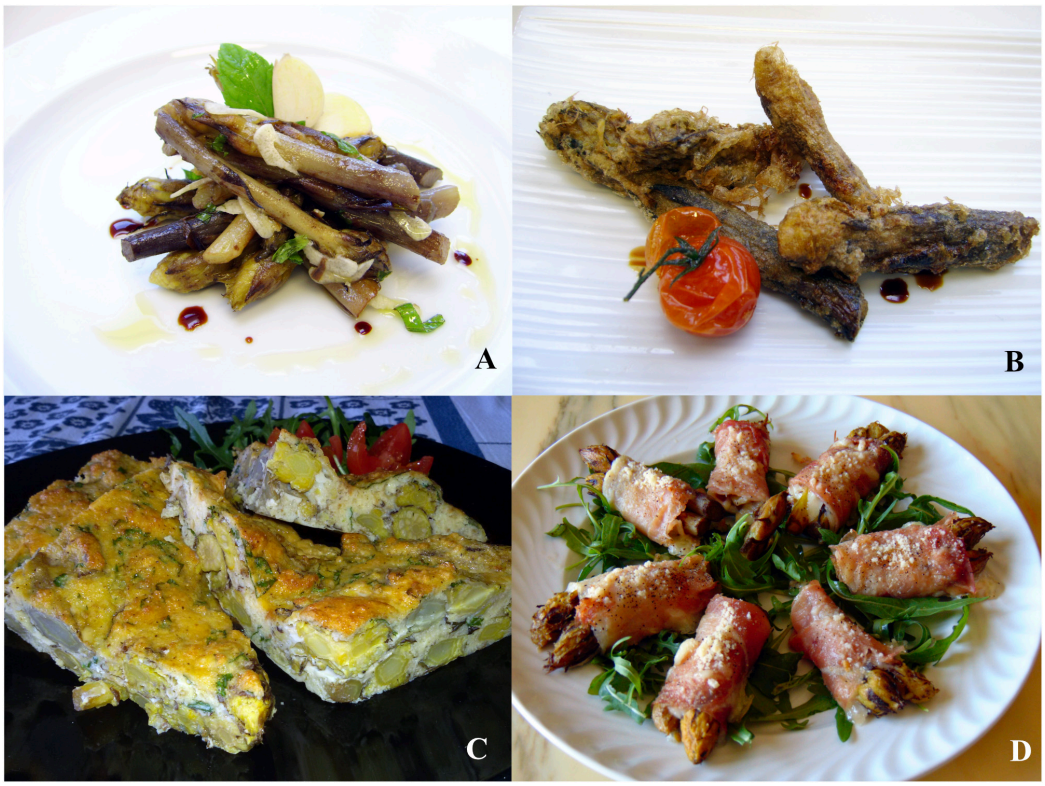

FIGURE 6 | Dishes based on crenate broomrape: salad with garlic, mint, vinegar and extra virgin olive oil **(A)**; floured and fried **(B)**; vegetable pie **(C)**; gratineéd ham roll **(D)**.

inflorescences of artichoke are a probable consequences of their different morphological and maturity traits. However, the results of the present study are comparable with the antioxidant activity of cardoon leaf stalks, as showed by Lahoz et al. (2011), who reported values in the range 2.63–12.12 meq Trolox 100 g^{-1} for eight cardoon (*C. cardunculus* L. var. *altilis* DC) cultivars.

Summer squash greens may be used in several Italian recipes like for other conventional leafy vegetables such as chicory and Swiss chard. Thus, it could be interesting to compare the proximate composition of these conventional vegetables with that of summer squash greens. The water content is relatively higher in summer squash greens respect to both chicory and Swiss chard. On the other hand, the content of protein, lipid and carbohydrate results lower in summer squash greens respect to both chicory and Swiss chard, while the content of dietary fiber is higher in summer squash greens (**Tables 1**, **3**). Moreover, it is important to highlight that summer squash greens show a relatively low content of sugars considering also that about 50% of the total amount is represented by fructose (**Table 2**), having a very low glycemic index. Therefore, considering both content and composition of all types of carbohydrates (including fiber), summer squash greens could be recommended as a vegetable to use especially in the case of hypoglycemic diets. Regarding the content of principal cations (**Table 2**) summer squash greens may be considered as very low contributors to the sodium supply, since a serving size of 100 g supplies about 1.1% of the Na daily intake (European Food Safety Authority, 2006). With regard to nitrate content (**Table 2**), summer squash greens can be considered as a vegetables with a very low content (<200 mg kg^{-1} FW) like for summer squash fruits as well as other vegetables such as green beans (*Phaseolus vulgaris* L.), melons (*C. melo* L.), tomatoes (*Solanum lycopersicum* L.), peppers (*Capsicum annuum* L.) and potatoes (*Solanum tuberosum* L.) (Santamaria, 2006). It is very important to highlight that to the best of our knowledge there are no other leafy and/or leafy-like vegetables with a similar low NO_3^- content. Therefore, these results suggest that summer squash greens should be recommended as a leafy vegetables to use especially for children's, since European Regulation 1258/2011 (European Commission, 2011) imposes a maximum nitrate concentration of 200 mg 100 kg^{-1} FW for children food products.

Similarly to what reported for summer squash greens, also faba greens are traditionally used in Puglia for different recipes including first courses and side dishes. Considering that the similarity of the faba greens to spinach leaves (*Spinacia oleracea* L.) has been a decisive factor in using this plant part as a food, it could be interesting to compare the proximate composition between these conventional and unconventional vegetables. Faba greens showed a lower water content respect to spinach leaves but a higher protein content (about threefold higher) and total carbohydrate including fiber (**Tables 1**, **3**). Regarding the content of principal cations (**Table 2**) faba greens may be considered as

TABLE 1 | Proximate composition of globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape.

	Globe artichoke's offshoots		Summer squash greens	Faba greens	Crenate broomrape	Significance
	Lucera	Locale di Mola				
Water	89.38c	91.48b	94.06a	84.77d	85.20d	***
Protein	2.41b	1.82bc	1.36c	5.35a	2.09b	***
Total lipid	0.30b	0.22b	0.06c	0.21b	0.40a	***
Total carbohydrate	6.44c	5.36c	3.01d	8.68b	11.46a	***
Fiber, total dietary	4.03a	3.14ab	1.88b	3.15ab	2.20b	***
Ashes	1.47a	1.11b	1.50a	1.00b	0.86b	***

*Mean values are expressed as g 100 g^{-1} FW. For each parameter, the same letters in the same row indicate that mean values are not significantly different (P = 0.05). Significance: ***P ≤ 0.001.*

TABLE 2 | Content of principal cations, nitrate and sugars in globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape.

	Globe artichoke's offshoots		Summer squash greens	Faba greens	Crenate broomrape	Significance
	Lucera	Locale di Mola				
Na^+	48.11b	159.52a	16.89c	21.35c	20.87c	***
K^+	489.76a	184.63c	488.88a	323.95b	252.95c	***
Mg^{2+}	29.23ab	27.68ab	38.41a	32.99ab	24.98b	*
Ca^{2+}	107.46a	91.31a	97.91a	63.33b	51.18b	***
NO_3^-	110.42a	86.11ab	13.03c	82.86a	50.92b	***
Glucose	767.4a	815.0a	107.1c	186.3c	477.3b	***
Fructose	315.3b	175.2c	134.3c	112.1c	995.2a	***
Sucrose	197.7b	89.0b	37.5b	446.6a	190.8b	**

*Mean values are expressed as mg 100 g^{-1} FW. For each parameter, the same letters in the same row indicate that mean values are not significantly different (P = 0.05). Significance: ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05.*

TABLE 3 | Proximate composition of cardoon, globe artichoke, leafy chicory, Swiss chard, spinach, and asparagus.

	Cardoons	Globe artichoke	Leafy chicory	Swiss chard	Spinach	Asparagus
Water	94.00	84.94	92.00	92.66	91.40	93.22
Protein	0.70	3.27	1.70	1.80	2.86	2.20
Total lipid	0.10	0.15	0.30	0.20	0.39	0.12
Total carbohydrate	4.07	10.51	4.70	3.74	3.63	3.88
Fiber, total dietary	1.06	5.40	0.70	1.60	2.20	2.10

Values are reported from the National Nutrient Database of the United States Department of Agriculture (United States Department of Agriculture [USDA], 2017).

very low contributors to the sodium supply, since a serving size of 100 g supplies about 1.4% of the daily intake (European Food Safety Authority, 2006). Regarding nitrate (**Table 2**) faba greens may be considered as vegetables with a middle content (range of 500–1000 mg kg^{-1} FW), similar to that observed in offshoots of *Locale di Mola* globe artichoke and other conventional vegetables (Santamaria, 2006). Instead, spinach is considered a vegetable with very high content of nitrate, since it contains more than 2500 mg $NO_3^-{\cdot}kg^{-1}$ FW (Santamaria, 2006). It is important to highlight that faba greens show also an interesting antioxidant activity, considering that a serving size of 100 g supply about 11,455 μmol of Trolox Equivalent (**Figure 7**). Therefore, the results of the present study suggest that faba greens may be considered as a very nutritious vegetable as well as a potential spinach substitute especially for its lower content of nitrate. Moreover, considering the use of faba greens also as a raw vegetable in salads (**Figure 5C**), it is possible to hypothesize the use of this unconventional vegetable also as a potential substitute of other vegetables with high NO_3^- content such as lettuce and rocket (Santamaria, 2006).

Because the resemblance of the young stems of *O. crenata* L. to large asparagus was a decisive factor in eating this parasitic plant, it could be interesting to compare the proximate composition between asparagus and crenate broomrape. The water content is lower in crenate broomrape, while the content of protein and lipid and fiber is relatively similar between the two vegetables. On the other hand, crenate broomrape shows a content of total carbohydrate about threefold higher respect to asparagus (**Tables 1**, **3**). Regarding the content of principal cations (**Table 2**) like for summer squash greens and faba greens, also crenate broomrape may be considered as a very low contributor to the sodium supply, since a serving size of 100 g supplies about

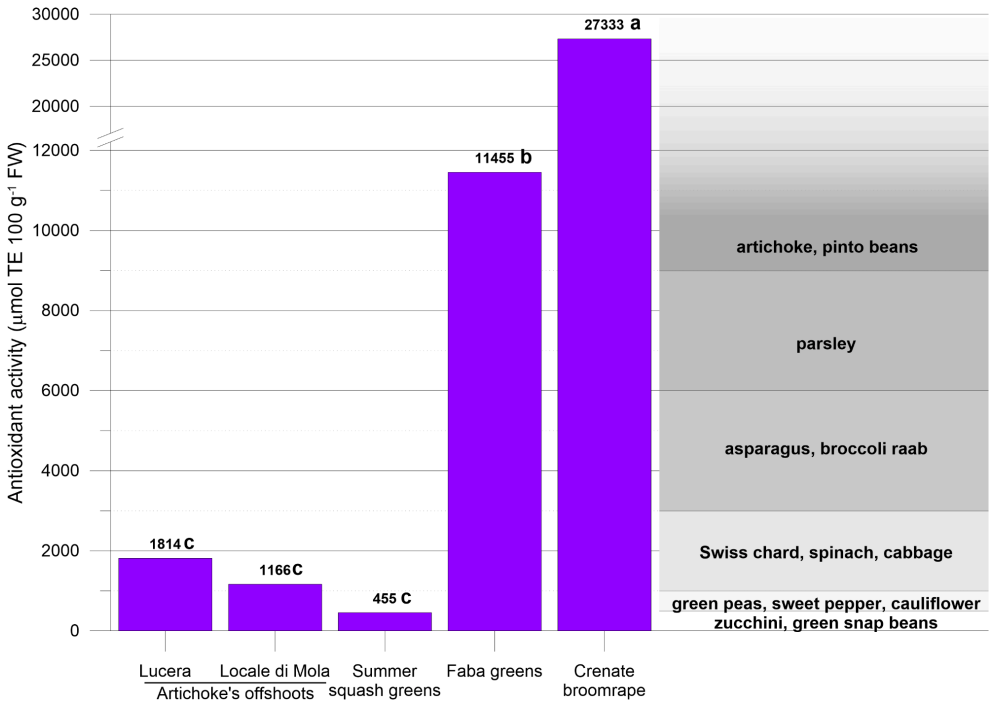

FIGURE 7 | Antioxidant activity of globe artichoke's offshoots, summer squash greens, faba greens and crenate broomrape, expressed as micromoles of Trolox Equivalent (TE) per 100 g of fresh weight (FW). Letters adjacent to data labels indicate statistical differences among the unconventional vegetables. The same letters indicate that mean values are not significantly different ($P = 0.05$). Typical antioxidant activity ranges for conventional vegetables are also reported according to Pennington and Fisher (2009).

1.4% of the daily intake (European Food Safety Authority, 2006). As for potassium, the results of the present study suggest that crenate broomrape may be considered as a low contributor to the daily supply of this cation, since 100 g of this unconventional vegetable could satisfy only about the 5.4% of daily adequate intake (Food and Nutritional Board, 2005). Regarding nitrate (**Table 2**) crenate broomrape may be considered a vegetable with a low-middle content (about 500 mg NO_3^- kg^{-1} FW) according to the classification proposed by Santamaria (2006), though NO_3^- content in crenate broomrape results higher respect to asparagus (Santamaria, 2006). It is important to underline that crenate broomrape shows a very interesting antioxidant activity, since a serving size of 100 g supply about 27,333 μmol of Trolox Equivalent (**Figure 7**). All these results suggest that crenate broomrape may be considered as a very nutritious Traditional Agri-Food Product of Puglia.

Finally, exploitation of offshoots of globe artichoke, summer squash greens, faba greens and crenate broomrape could be carried out through several initiatives, they integrating into the potential multi-functionality of farms. Thus, the exploitation of these unconventional vegetables may require a multi-disciplinary approach and integrated projects for protecting farmers communities and promoting the artisanal agri-food products. For example, the obtainment of labeled products such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialties Guaranteed (TSG) could be a good opportunity.

CONCLUSION

Offshoots of globe artichoke, summer squash greens, faba greens, and crenate broomrape have good potential as novel foods, being nutritious and refined products. Therefore, the exploitation of these unconventional vegetables may satisfy the needs of specific markets characterized by a demand for local and sustainable food products. On the other hand, the culinary use of these unconventional vegetables as well as their interesting quality traits are known only in some local areas. Therefore, specific activities are needed in order to disseminate knowledge, promote quality and boost consumer demand. Finally, offshoots of globe artichoke, summer squash greens and crenate broomrape require a long time before to be used (especially cleaning-up and soaking) and this may dissuade potential consumers. Therefore, new research should be carried out for obtaining new potential ready-to-eat retail products such as cook-chilled vegetables that may be an innovative and appealing idea for consumers.

AUTHOR CONTRIBUTIONS

MR: substantial contributions to the conception or design of the work, drafting the work, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated

and resolved. AS: revised the article critically, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. VP: performed the proximate analysis, interpretation of data, Revised the article critically, and final approval of the version to be published. PS: substantial contributions to the conception or design of the work, analysis and interpretation of data, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This research was supported by Regione Puglia Administration under Rural Development Program 2014–2020 – Project 'Biodiversity of vegetable crops in Puglia (BiodiverSO)', Measure 10, Sub measure 10.2, Operation 1 "Program for the conservation and the valorization of the genetic resources in agriculture", and "Intervento cofinanziato dal Fondo di Sviluppo e Coesione 2007–2013, APQ Ricerca Regione Puglia – Programma regionale a sostegno della specializzazione intelligente e della sostenibilità sociale ed ambientale FutureInResearch" – Project 'Innovazioni di prodotto e di processo per la valorizzazione della Biodiversità Orticola pugliese (InnoBiOrt)'.

ACKNOWLEDGMENTS

The authors thank Maria Castellino, Beniamino Leoni and Gerolmina Florio for technical assistance and Nicola Gentile, Francesco Di Giovine, Riccardo Gammino and Domenica Rutigliano for samples and photo collection.

REFERENCES

AOAC (1991). *Official Methods of Analysis*, 16th Edn. Washington, DC: Association of Official Analytical Chemists.

Bianco, V. V., Mariani, R., and Santamaria, P. (2009). *Piante Spontanee nella Cucina Tradizionale Molese*. Bari: Levante Editore.

Bianco, V. V., Santamaria, P., and Elia, A. (1998). Nutritional value and nitrate content in edible wild species used in southern Italy. *Acta Hortic.* 467, 71–90. doi: 10.17660/ActaHortic.1998.467.7

Cefola, M., Pace, B., Renna, M., Santamaria, P., Signore, A., and Serio, F. (2012). Compositional analysis and antioxidant profile of yellow, orange and purple *Polignano carrots*. *Ital. J. Food Sci.* 24, 284–291.

D'Acunzo, F., Giannino, D., Longo, V., Ciardi, M., Testone, G., Mele, G., et al. (2017). Influence of cultivation sites on sterol, nitrate, total phenolic contents and antioxidant activity in endive and stem chicory edible products. *Int. J. Food Sci. Nutr.* 68, 52–64. doi: 10.1080/09637486.2016.1221386

Difonzo, G., Russo, A., Trani, A., Paradiso, V. M., Ranieri, M., Pasqualone, A., et al. (2017). Green extracts from Coratina olive cultivar leaves: antioxidant characterization and biological activity. *J. Funct. Foods* 31, 63–70. doi: 10.1016/j.jff.2017.01.039

D'Imperio, M., Renna, M., Cardinali, A., Buttaro, D., Santamaria, P., and Serio, F. (2016). Silicon biofortification of leafy vegetables and its bioaccessibility in the edible parts. *J. Sci. Food Agric.* 96, 751–756. doi: 10.1002/jsfa.7142

Elia, A., and Santamaria, P. (2013). Biodiversity in vegetable crops, a heritage to save: the case of Puglia region. *Ital. J. Agron.* 8, 21–34. doi: 10.4081/ija.2013.e4

European Commission (2011). Commission regulation (EU) No 1258/2011 of 2 December 2011 amending regulation (EC) No 1881/2006 as regards maximum levels for nitrates in foodstuffs. *Off. J. Eur. Union* L320, 15–17.

European Food Safety Authority (2006). *Tolerable Upper Intake Levels for Vitamins and Minerals*. Available at: http://www.efsa.europa.eu/it/ndatopics/docs/ndatolerableuil.pdf

Flamm, G., Glinsmann, W., Kritchevsky, D., Prosky, L., and Roberfroid, M. (2001). Inulin and oligofructose as dietary fiber: a review of the evidence. *Crit. Rev. Food Sci. Nutr.* 41, 353–362. doi: 10.1080/20014091091841

Food and Nutritional Board (2005). *Dietary Reference Intakes for Water, Potassium, Sodium, Chloride, and Sulfate*. Washington, DC: The National Academies Press.

Gramignani, E. (1934). *Trattato di Orticoltura*. Avellino: Tip. C. Labruna.

Ingrassia, G. F. (1576). *Informatione del Pestifero, et Contagioso Morbo*. Palermo: Giouan Mattheo Mayda.

Lahoz, I., Fernández, J. A., Migliaro, D., Macua, J. I., and Egea-Gilabert, C. (2011). Using molecular markers, nutritional traits and field performance data to characterize cultivated cardoon germplasm resources. *Sci. Hortic.* 127, 188–197. doi: 10.1016/j.scienta.2010.10.002

Lattanzio, V., Kroon, P., Linsalata, V., and Cardinali, A. (2009). Globe artichoke: a functional food and source of nutraceutical ingredients. *J. Funct. Foods* 1, 131–144. doi: 10.1016/j.jff.2009.01.002

Margaroli, G. B. (1831). *Manuale dell'Abitatore di Campagna e della Buon Castalda*. Milano: Luigi Nervetti Tipografo-Libraio.

Matthioli, M. P. A. (1613). *I Discorsi di M. Pietro Maria Matthioli nei sei Libri di Pedacio Dioscoride Anazarbeo*. Venetia: Appresso Vincenzo Malpighi.

Pennington, J. A., and Fisher, R. A. (2009). Classification of fruits and vegetables. *J. Food Compos. Anal.* 22, S23–S31. doi: 10.1016/j.jfca.2008.11.012

Perrino, P., Laghetti, G., and Terzi, M. (2004). Modern concepts for the sustainable use of plant genetic resources in the Mediterranean natural protected areas: the case study of the Alta Murgia Park (Italy). *Genet. Resour. Crop. Evol.* 53, 695–710. doi: 10.1007/s10722-004-3942-x

Prosky, L., and Hoebregs, H. (1999). Nutritional and health benefits of inulin and oligofructose methods to determine food inulin and oligofructose. *J. Nutr.* 129, 1418–1423. doi: 10.1093/jn/129.7.1418S

Renna, M. (2015). "From the farm to the plate": agro-biodiversity valorization as a tool for promoting a sustainable diet. *Prog. Nutr.* 17, 77–80.

Renna, M., Gonnella, M., Giannino, D., and Santamaria, P. (2014a). Quality evaluation of cook-chilled chicory stems (*Cichorium intybus* L., Catalogna group) by conventional and sous vide cooking methods. *J. Sci. Food Agric.* 94, 656–665. doi: 10.1002/jsfa.6302

Renna, M., Rinaldi, V. A., and Gonnella, M. (2015a). The Mediterranean Diet between traditional foods and human health: the culinary example of Puglia (Southern Italy). *Int. J. Gastron. Food Sci.* 2, 63–71. doi: 10.1016/j.ijgfs.2014.12.001

Renna, M., and Santamaria, P. (2017). Presi in ortaggio. Otto prodotti straordinari della biodiversità pugliese. Bari: Università degli Studi di Bari Aldo Moro.

Renna, M., Serio, F., and Santamaria, P. (2015b). Crenate broomrape (*Orobanche crenata* Forskal): prospects as a food product for human nutrition. *Genet. Resour. Crop Evol.* 62, 795–802. doi:10.1007/s10722-015-0249-z

Renna, M., Serio, F., Signore, A., and Santamaria, P. (2014b). The yellow–purple Polignano carrot (*Daucus carota* L.): a multicoloured landrace from the Puglia region (Southern Italy) at risk of genetic erosion. *Genet. Resour. Crop Evol.* 61, 1611–1619. doi: 10.1007/s10722-014-0155-9

Ronkart, S. N., Blecker, C. S., Fourmanoir, H., Fougnies, C., Deroanne, C., Van Herck, J. C., et al. (2007). Isolation and identification of inulooligosaccharides resulting from inulin hydrolysis. *Anal. Chim. Acta* 604, 81–87. doi: 10.1016/j.aca.2007.07.073

Sada, L. (1991). *La Cucina in Terra di Bari*. Padova: Franco Muzzio Editore.

Santamaria, P. (2006). Nitrate in vegetables: toxicity, content, intake and EC regulation. *J. Sci. Food Agric.* 86, 10–17. doi: 10.1002/jsfa.2351

Serio, F., Florio, G., Parente, A., and Santamaria, P. (2005). Confronto tra sistemi di coltivazione di carosello e barattiere in serra. II. Aspetti qualitativi. *Colture Protette* 34(Suppl. 5), S28–S35.

Signore, A., Serio, F., and Santamaria, P. (2014). Wikipedia as a tool for disseminating knowledge of (agro) biodiversity. *HortTechnology* 24, 118–126.

Soderini, G. V. (1824). *Della Cultura degli orti e Giardini*. Firenze: Stamperia del Giglio.

Tamaro, D. (1937). *Ortaggi di Grande Reddito. Trattato di Orticoltura Industriale*, Vol. II. Milano: Ulrico Hoepli.

Testone, G., Mele, G., Di Giacomo, E., Gonnella, M., Renna, M., Tenore, G. C., et al. (2016). Insights into the sesquiterpenoid pathway by metabolic profiling and *de novo* transcriptome assembly of stem-chicory (*Cichorium intybus* Cultigroup "Catalogna"). *Front. Plant Sci.* 7:1676. doi: 10.3389/fpls.2016.01676

Turchi, A. (1957). *Orticoltura pratica*. Bologna: Edizioni agricole.

United States Department of Agriculture [USDA] (2017). *National Nutrient Database for Standard Reference Release 28*. Available at: https://goo.gl/8J2jCt

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.

The reviewer SP and handling Editor declared their shared affiliation.

frontiers in Plant Science

MINI REVIEW
published: 15 August 2018
doi: 10.3389/fpls.2018.01199

Orphan Legumes Growing in Dry Environments: Marama Bean as a Case Study

Christopher Cullis[1], Percy Chimwamurombe[2], Nigel Barker[3], Karl Kunert[4] and Juan Vorster[4]*

[1] Department of Biology, Case Western Reserve University, Cleveland, OH, United States, [2] Department of Natural and Applied Sciences, Namibia University of Science and Technology, Windhoek, Namibia, [3] Department of Plant and Soil Sciences, University of Pretoria, Pretoria, South Africa, [4] Department of Plant and Soil Sciences, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

Plants have developed morphological, physiological, biochemical, cellular, and molecular mechanisms to survive in drought-stricken environments with little or no water caused by below-average precipitation. In this mini-review, we highlight the characteristics that allows marama bean [*Tylosema esculentum* (Burchell) Schreiber], an example of an orphan legume native to arid regions of southwestern Southern Africa, to flourish under an inhospitable climate and dry soil conditions where no other agricultural crop competes in this agro-ecological zone. Orphan legumes are often better suited to withstand such harsh growth environments due to development of survival strategies using a combination of different traits and responses. Recent findings on questions on marama bean speciation, hybridization, population dynamics, and the evolutionary history of the bean and mechanisms by which the bean is able to extract and conserve water and nutrients from its environment as well as aspects of morphological and physiological adaptation will be reviewed. The importance of the soil microbiome and the genetic diversity in this species, and their interplay, as a reservoir for improvement will also be considered. In particular, the application of the newly established marama bean genome sequence will facilitate both the identification of important genes involved in the interaction with the soil microbiome and the identification of the diversity within the wild germplasm for genes involved drought tolerance. Since predicted future changes in climatic conditions, with less water availability for plant growth, will severely affect agricultural productivity, an understanding of the mechanisms of unique adaptations in marama bean to such conditions may also provide insights as to how to improve the performance of the major crops.

Keywords: orphan legumes, marama bean, drought response, soil microbiome, genome sequence

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Nicolas Rispail, Consejo Superior de Investigaciones Científicas (CSIC), Spain
Francesca Sparvoli, Istituto di Biologia e Biotecnologia Agraria (IBBA), Italy

****Correspondence:***
Karl Kunert
karl.kunert@up.ac.za

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *04 June 2018*
Accepted: *26 July 2018*
Published: *15 August 2018*

Citation:
Cullis C, Chimwamurombe P, Barker N, Kunert K and Vorster J (2018) Orphan Legumes Growing in Dry Environments: Marama Bean as a Case Study. Front. Plant Sci. 9:1199. doi: 10.3389/fpls.2018.01199

DRY ENVIRONMENTS AND ORPHAN LEGUMES

Dry environments have little or no water with below-average precipitation due to periodic droughts resulting in prolonged water shortage. A drought period can last from a few days to months or years. Such a drought period is often accompanied by intensive heat significantly worsening drought effects due to additional increased water evaporation. Drought-stricken environments

are highly unsuitable for production of high-input, high-yielding food crops mostly selected for optimal yield under non-drought conditions (Evenson and Gollin, 2003).

Orphan, or underutilized, legumes, are staple food crops in many developing countries. Their neglect has been the subject of two reports from the National Research Council (1979, 2006). They have generally little economic importance and have not been greatly improved by breeders (Naylor et al., 2004; Foyer et al., 2016). Like landraces of major food crops, orphan legumes have genetically adapted over time to their natural environment largely unmodified by human breeding efforts (Trytsman et al., 2011). These legumes are often better suited to withstand harsh growth environments, having developed morphological, physiological, biochemical, cellular and molecular mechanisms to survive in dry drought-stricken environments (Osakabe et al., 2014; Tátrai et al., 2016). Although orphan legumes frequently respond like most plants to stress, they might have developed unique survival mechanisms using a combination of different traits and responses resulting in growth in dry drought-stricken environments. Detailed knowledge of such mechanisms therefore provides valuable information for breeders on useful traits and responses for survival in extreme environments (Cullis and Kunert, 2017). Such orphan legume crops include groundnut (*Arachis hypogaea*), grass pea (*Lathyrus sativus*), bambara groundnut (*Vigna subterranea*), cowpea (*Vigna unguiculata*), and marama bean (*Tylosema esculentum*), the last included by The Kirkhouse Trust focusing on improving locally important legume crops[1].

Purpose of this mini-review is to provide a short overview of the existing knowledge and current advances in the research to understand the biology of the plant. This also includes the mechanisms the bean employs to survive in regions where few conventional crops can thrive and flourish. An improved understanding of these mechanisms might also not only help breeders to learn more about particular characteristics for survival in dry drought stricken environments but also the bean's usefulness, both as a model and a future crop, to combat climate change and to attract interest in the development of the bean into mainstream agriculture.

MARAMA BEAN, A PEA FAMILY MEMBER

Marama bean, a wild perennial legume native to the Kalahari Desert in Southern Africa, grows mostly in sandy soils. Reports of the National Research Council, 1979 on "Tropical Legumes: Resources for the Future" and also in 2006 on "Lost Crops of Africa: Volume II: Vegetables" highlight the bean. In 1979, it was for example noted that "Of all the plants described in this book, the marama bean is, perhaps, the least developed," while in 2006 it was noted "Strange that marama has not been introduced into cultivation since above ground, this plant produces seeds that rival peanut and soybean in composition and nutritive value, and below, it produces a high-protein tuber much bigger and more nutritious than any potato, yam, or even sugar beet. In addition, it thrives in poor-quality soil and under the harshest of climates. Little is known about the plant and almost nothing is understood about its cultivation. Among Africa's many native foods, this remains one of the most neglected."

The bean is an important local dietary component, due to its high seed protein and high carbohydrate content of the tuberous root (**Figure 1**). In its native habitat, the bean withstands summer temperatures reaching 50°C with surface water available usually only for 8 weeks/year (Powell, 1987; Bower et al., 1988; Nepolo et al., 2009). Marama bean is currently being developed into a local crop (Chimwamurombe, 2011) and test gardens have been already established and efforts are undertaken in Namibia to produce the bean in well-fenced prepared land for local communities.

Marama bean belongs to the subfamily Cercidoideae of the Fabaceae (pea family). The genus *Tylosema*, to which the bean belongs, has four additional species (Lewis et al., 2005; Azani et al., 2017). All members of the Cercidoideae subfamily lack root nodules. *Tylosema* species have been investigated by both palynological and molecular analyses particularly by application with chloroplast markers such as the matK gene (Hao et al., 2003; Banks et al., 2013). A further member of the subfamily Cercidoideae is *Bauhinia*, a genus of more than 500 species of flowering plants widely cultivated as ornamental trees in tropical Asia. *Tylosema* was not considered as a distinct genus and was originally classified within the *Bauhinia* genus (Hao et al., 2003). However, *Tylosema,* was later recognized as a separate clade within the *Bauhiniinae* (Wunderlin, 2010). Separation between *Tylosema* and *Bauhinia* has recently been confirmed by complete chloroplast genomic sequencing and by comparing specific sequences of a number of genes both from the chloroplast and nucleus (Kim and Cullis, 2017). *T. esculentum*, *Tylosema fassoglense*, and putative Angolan species (Castro et al., 2005) may be, in reality, a single genetically diverse species, within which it maybe be possible to identify land races or some similar entity. As with many African plant lineages, marama bean fits nicely with the little used "ochlospecies" concept first proposed by White (1998), and which seems to apply to many widespread African taxa (Cronk, 1998).

DROUGHT ADAPTATION MECHANISMS

Marama bean has developed, as a drought avoider, several survival mechanisms for life in a dry drought-stricken environment. The morphological and physiological adaptation of the bean to its growth environment has been recently reviewed by Lawlor (2018). The bean reacts like many other plants growing in dry environments but combines different well-known avoidance mechanisms. The bean avoids water stress by leaf reduction to reduce water loss and reduces stem elongation and number of leaves, at the extreme to complete die back in cooler months (Mitchell et al., 2005; Travlos and Karamanos, 2008; Karamanos and Travlos, 2012). Under well-watered conditions, the bean is highly branched with runners extending along the ground and produces a great number of leaves and biomass

[1]http://www.kirkhousetrust.org/orphanlegumes.html#.V0AvnfmLTIU

FIGURE 1 | (A) Prostrate vines with flowers. **(B)** Young and edible marama bean tuberous roots. Scale bar in **A** is 10 cm. **(C)** Large tuber weighing approximately 240 kg, with abundant foliage. Scale bar in **C** is 25 cm. **(D)** Seeds. Scale bar in **D** is 1 cm.

(Mitchell et al., 2005; Karamanos and Travlos, 2012; **Figure 1A**). The bean has also a tap root allowing penetration deep below the surface to access subsoil-moisture (Comas et al., 2013). Since the bean grows in sandy soils, water can remain in the root zone for months after rain and a tap root is able to access this water.

Marama bean is also a creeper with scandent stems creeping in several directions covering large areas (Keegan and Van Staden, 1981; **Figure 1A**). This behavior very likely helps to withstand drying winds. Also, typical for a drought avoider, is the relatively early closure of stomata under water stress to save water and maintain the leaf water potential (Mitchell et al., 2005; Karamanos and Travlos, 2012). Stomatal opening represents an important regulatory mechanism during limited water supply and heat stress, influencing simultaneously water loss via transpiration and CO_2 diffusion into the leaf apoplast. This behavior is very different from a legume like soybean, in which large differences in leaf water potential exists between drought-stressed and unstressed leaves (Villalobos-Rodriquez and Shibles, 1985). Experiments have also been carried out to understand leaf movements in marama bean (Travlos et al., 2008). To avoid direct sunlight, plants can carry out complex daily heliotropic adjustments of leaf angles to reduce transpiration losses by diminishing the light interception (para-heliotropism) in which the *DREB1A* gene might play an important role (van Zanten et al., 2010; Rakocevic et al., 2017). Travlos et al. (2008) reported that the leaves of the bean are open during the day and close during the night, with similar behavior of plants under different growth temperatures. Potassium deficiency can, however, prevent leaf closure during the night but detailed investigations to determine if any heliotropic adjustments are also involved in drought avoidance are so far missing.

A further drought avoidance mechanism of the bean is the formation of a tuberous root (**Figures 1B,C**). The tuber root is both a starch reservoir and a water reservoir. The marama bean root is also high in protein containing about nine percent protein on a dry-weight basis (National Research Council, 2006). A large number of tropical legumes develop below-ground organs for carbohydrate storage (Saxon, 1981). The storage organ may be an enlargement of the tap root, swollen fibrous roots or, as in the case of marama bean, a true tuber. The bean very likely survives a drought condition by accessing the water stored in the tuber. Older tubers weighing more than 200 kg (**Figure 1C**) can contain 90% water by weight. Since the tuber remains viable under drought, it allows rapid vegetative re-growth of stems under more favorable growth conditions as a drought survival strategy (Travlos and Karamanos, 2008). Few older leaves may

also maintain leaf function under drought to allow more rapid re-growth when water is again available (Mitchell et al., 2005). However, when the water content of the tuber falls to about 85%, these older leaves are discarded to increase survival for months in a dry environment.

The tuber also has potential as a component of yield. Since marama bean does not flower until the second year after planting from seed, the tuber, if well-developed, can be harvested as a carbohydrate source as well as for starch with interesting properties (Adeboye and Emmambux, 2017; Nyembwe et al., 2018). No detailed information on tuber development is currently available. A different breeding strategy may also be necessary for identifying rapidly developing tubers for harvest as an annual domesticated crop. This also includes a more detailed study on variation in tuber development.

The lack of any root nodules to fix atmospheric nitrogen in marama bean (Dakora et al., 1999) may also have helped evolving in a dry drought-stricken environment. The whole genome assembly from Illumina and PacBio shotgun sequencing has facilitated identification of the marama leghemoglobin genes. These genes are more closely related to the non-symbiotic plant hemoglobin genes rather than the leghemoglobin from symbiotic legumes (Cullis, 2017) supporting an evolutionary hypothesis. Legumes are able to form such root nodules for biological nitrogen fixation facilitated through symbiotic interaction with rhizobia. Although nodulation and atmospheric nitrogen fixation provides a benefit for growth (Morgan et al., 2005), lack of both might be a potential enhancement of drought avoidance. The lack of root nodules avoids dependence on a nitrogen source highly affected by drought conditions. Drought sensitivity of nodules and the particular negative effects on the nodulation process is well known (Serraj et al., 1999; Gil-Quintana et al., 2013). Drought particularly affects supply of photosynthate to nodules, required for symbiotic nitrogen fixation, and also impairs nitrogenase activity with breakdown of the oxygen diffusion barrier and loss of leghemoglobin (King and Purcell, 2006; Arrese-Igor et al., 2011).

MARAMA BEAN MICROBIOTA

Legumes are generally not highly dependent on atmospheric nitrogen fixation, with both soybean and faba bean the exceptions (Peoples et al., 2009). Legumes can use alternative organic soil nitrogen sources. Soil nitrogen might, for example, derive from compounds such as amino acids (Cao et al., 2016). Such amino acids are found in the rhizosphere as a result of lysis of cells from plants and microbes. Initial experiments to investigate the exact type of soil-derived nitrogen for marama bean have recently been done. Several bacteria have been so far isolated from the rhizosphere of arid-adapted marama bean plants (Kandjimi et al., 2015). All isolates were able to produce ammonia in plate assays. Although ammonia released by bacteria might be a nitrogen source, more information is required about ammonia tolerance of the bean. Plants generally tolerate only low levels of ammonia (Weise et al., 2013). The soil microbiome associated with the bean growing in different regions of Namibia is currently being characterized through population sequencing of the bacterial 16S, V3–V4 region and fungal ITS 1 regions. This will also add to the initial characterization of the endophytes that can be harbored by bean seeds potentially contributing to their nutritional efficiency since they have a striking capacity to harness nitrogen into seed protein (Chimwamurombe et al., 2016). Mycorrhizal fungi are further characterized in the microbiome. These fungi can alleviate drought stress in plants through both tolerance and avoidance mechanisms (Finlay et al., 2008; Rapparini and Peñuelas, 2013). The enhancement of tolerance of plants to water deficit by mycorrhizae may particularly involve the regulation of drought-induced plant genes, such as aquaporins, both by the down-regulation of genes encoding plasma membrane aquaporins (Porcel et al., 2006) or the enhanced expression of specific aquaporins (Li et al., 2012).

AREAS REQUIRING FURTHER EXPLORATION

Knowledge about avoidance mechanisms in marama bean, but also in other orphan legumes, is still scanty and fragmented and requires more research efforts. Such efforts will not only further drive marama bean breeding but also allow the identification of any correct combination of traits and responses for better survival of a crop in a dry drought-stricken environment. Interesting questions are, for example, why the bean has relatively large leaves (possibly to carry out photosynthesis in a relatively short time) and how the bean maintains, with large leaves, a leaf temperature allowing metabolic processes with Rubisco activase – a key enzyme in keeping the Calvin cycle functional – particularly heat-sensitive (Feller and Vaseva, 2014). This paradox of existence of large-leaf species in deserts, instead of species with small leaves for better reducing any water loss and also intercepting less radiation, is not entirely understood. High rates of leaf transpiration likely provides a significant cooling effect and leaf transpiration is possibly an important trait for surviving in dry and hot environments (Smith, 1978; Chaves et al., 2016; Lin et al., 2017). However, whether this also applies to marama bean has still to be shown.

A further important question is whether marama bean can also use, in addition to drought avoidance, drought tolerance mechanisms with expression of genes providing cellular protection against drought exposure with better water accumulation or can even use drought escape mechanisms (Feller and Vaseva, 2014; Fang and Xiong, 2015; Shavrukov et al., 2017). In this regard, the bean shows no sign of enhanced photosynthetic water-use efficiency at the level of leaf photosynthesis when compared with other well-characterized C3 plants. Rubisco kinetics are further consistent with adaptation to hot, drought-prone environments (Mitchell et al., 2005). Any transcriptomic analysis in the bean is so far also missing to determine, for example, if, and how, genes known to be involved in antioxidant and osmolyte production for drought tolerance are possibly expressed (Hayat et al., 2012;

Das and Roychoudhury, 2014). Karamanos and Travlos (2012) found, however, already evidence for a possible progressive osmotic adjustment being more intense in older plants. More negative values of solute potential at zero turgor were thereby considered as an ability of osmotic adjustment through the production, or accumulation, of compatible osmolytes with mobilization of osmotical substances like sugars from the carbohydrates stored in the marama bean root. The Cullis group has therefore recently established a genomic database by high-throughput next-generation sequencing with data from both short read Illumina platform as well as long read PacBio platform. This database, a significant resource in the search for and expression of known protective proteins, now allows the identification and isolation of particular genes known to be involved in drought tolerance. However, an important question remains what benefit a drought tolerance mechanism will have to a plant like marama bean when growing in an dry environment exposed to lengthy drought periods and extensive heat?

A further interesting research topic will be the identification of the actual nitrogen source for the bean. Future bioinformatic studies should therefore also focus on other components of the symbiotic nitrogen fixing pathway to determine whether or not it would be possible for the bean to develop this activity. Symbiosis-related genes might also inform on possible pathways for other bean–microbial interactions in association with information from soil microbiome studies, since there are parallels in the pathways for symbiosis development and mycorrhizal associations. Characterization of the bean's soil microbiome is consequently an important task, both for nutritional and stress tolerance characters. Interrogation of the genome database for genes necessary for nodulation should further be directed toward identifying rhizobia initiating bean nodulation (De Souza et al., 2015). Finding the nitrogen source(s) for marama bean might also be of relevance for generally growing legumes in dry drought-stricken environments where nodulation for atmospheric nitrogen fixation is severely affected by drought and nitrogen fertilization too costly. Future research also needs to determine if the bean is using a single nitrogen source, such as microbe-derived ammonia, or multiple sources including non-microbial sources. A more detailed investigation of the marama bean microbiome, currently carried out in Namibia and the United States, is therefore an essential task in identification of such nitrogen sources. Marama bean, with its apparently efficient acquisition of nutrition from poor arid soils, might ultimately also provide insights into developing useful microbial fertilizers, especially adapted to arid environments (Chimwamurombe et al., 2016).

Finally, marama bean is an obligate outcrossing species that appears to be functionally hexaploid. Characterization of variation within the germplasm and its relationship to the mechanisms of coping with drought will also identify which genes are important for these processes. Diversity of the bean also indicates that there are possibly differences in growth rate and tuber formation and size. Future identification of interplay of the genome with the microbial associated population will also inform how important relevant genetic variation will be. It has already been shown that the chloroplast genome is variable with individual bean plants heteroplasmic (Kim and Cullis, 2017). Has variability also any relevance to adaptation to a harsh environment is thereby also an important question to answer. Availability of many whole genome sequences for characterized individuals from different environments and the associated microbiome will allow identification of survival/flourishing strategies adopted by the bean and, perhaps, point a way to improving drought tolerance in other crop species.

AUTHOR CONTRIBUTIONS

KK and CC developed the first draft to which PC, NB, and JV added to and edited the text. All the authors approved the final submission.

FUNDING

NB was funded for this project through a University of Pretoria Research Development Program grant; KK and JV by NRF incentive funding; CC from the Oglebay fund; and PC from the Kirkhouse Trust.

REFERENCES

Adeboye, A. S., and Emmambux, N. M. (2017). Physicochemical, morphological, thermal and pasting properties of marama (*Tylosema esculentum*) storage root starch. *Stärke* 69, 3–4. doi: 10.1002/star.201600084

Arrese-Igor, C., González, E. M., Marino, D., Ladrera, R., Larrainzar, E., and Gil-Quintana, E. (2011). Physiological response of legumes nodules to drought. *Plant Stress* 5, 24–31.

Azani, N., Babineau, M., Donovan Bailey, C., Banks, H., Barbosa, A. R., and Pinto, R. B. et al. (2017). A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny. *Legume Phylogeny Work. Group* 66, 44–77. doi: 10.12705/661.3

Banks, H., Forest, F., and Lewis, G. (2013). Palynological contribution to the systematics and taxonomy of Bauhinia s.l. (Leguminosae: Cercideae). *S. Afr. J. Bot.* 89, 219–226. doi: 10.1016/j.sajb.2013.07.028

Bower, N., Hertel, K., Oh, J., and Storey, R. (1988). Nutritional-evaluation of marama bean (*Tylosema esculentum*, fabaceae): analysis of the seed. *Econ. Bot.* 42, 533–540. doi: 10.1007/BF02862798

Cao, X., Ma, Q., Zhong, C, Yang, X, Zhu, L., and Zhang, J., et al. (2016). Elevational variation in soil amino acid and inorganic nitrogen concentrations in Taibai Mountain, China. *PLoS One.* 11:e0157979. doi: 10.1371/journal.pone.0157979

Castro, S., Silveira, P., Coutinho, A. P., and Figueiredo, E. (2005). Systematic studies in Tylosema (Leguminosae). *Bot. J. Linnean Soc.* 147, 99–115. doi: 10.1016/j.phytochem.2015.09.004

Chaves, M. M., Costa, J. M., Zarrouk, O., Pinheiro, C., Lopes, C. M., and Pereira, J. S. (2016). Controlling stomatal aperture in semi-arid regions—the dilemma of saving water or being cool? *Plant Sci.* 251, 54–64. doi: 10.1016/j.plantsci.2016.06.015

Chimwamurombe, P. M. (2011). Domestication of [*Tylosema esculentum* (Burchell) schreiber] (Marama bean): a work in progress in Namibia. *Biosci. Biotech. Res. Asia.* 8, 549–556. doi: 10.13005/bbra/898

Chimwamurombe, P. M., Grönemeyer, J. M., and Reinhold-Hurek, B. (2016). Isolation and characterization of culturable seed-associated bacterial endophytes from gnotobiotically grown Marama bean seedlings *FEMS Microbiol. Ecol.* 92:fiw083. doi: 10.1093/femsec/fiw083

Comas, L. H., Becker, S. R., Cruz, V. M. V., Byrne, P. F., and Dierig, D. A. (2013). Root traits contributing to plant productivity under drought. *Front. Plant Sci.* 4:442. doi: 10.3389/fpls.2013.00442

Cronk, Q. C. B. (1998). "The ochlospecies concept," in *Chorology, Taxonomy and Ecology of the Floras of Africa and Madagascar,* eds Huxley, C. R., Lock, J. M., Cutler, D. F (Kew: Royal Botanic Gardens), 155–170.

Cullis, C., and Kunert, K. J. (2017). Unlocking the potential of orphan legumes, *J. Exp. Bot.* 68, 1895–1903. doi: 10.1093/jxb/erw437

Cullis, C. A. (2017). A novel inversion and heteroplasmy in the chloroplast genome of Marama (*Tylosema esculentum*). *Int. Bot. Conf.* 68, 2065–2072. doi: 10.1093/jxb/erw500

Dakora, F. D., Lawlor, D. W., and Sibuga, K. P. (1999). Assessment of symbiotic nitrogen nutrition in marama bean (*Tylosema esculentum* L.) a tuber-producing underutilized African grain legume. *Symbiosis* 27, 269–277.

Das, K., and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front. Environ. Sci.* 2:53. doi: 10.3389/fenvs.2014.00053

De Souza, R., Ambrosini, A., and Passaglia, L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils Genet. *Mol. Biol.* 38, 401–419. doi: 10.1590/S1415-475738420150053

Evenson, R. E., and Gollin, D. (2003). Assessing the impact of the green revolution, 1960 to 2000. *Science* 300, 758–762. doi: 10.1126/science.1078710

Fang, Y., and Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* 72, 673–689. doi: 10.1007/s00018-014-1767-0

Feller, U., and Vaseva, I. I. (2014). Extreme climatic events: impacts of drought and high temperature on physiological processes in agronomically important plants. *Front. Environ. Sci.* 2:39. doi: 10.3389/fenvs.2014.00039

Finlay, R. D., Lindahl, B. D., Taylor, A. F. S. (2008). "Responses of mycorrhizal fungi to stress," in *Stress in Yeasts and Filamentous Fungi*, eds Avery S, Stratford M, and van West P, (Amsterdam: Elsevier), 201–220. doi: 10.3389/fenvs.2014.00039

Foyer, C. H., Lam, H-M., Nguyen, H. T., Siddique, K. H. M., Varshney, R., and Colmer, T. D. et al. (2016). Neglecting legumes has compromised global food and nutritional security. *Nat. Plants* 2:16112. doi: 10.1038/nplants.2016.112

Gil-Quintana, E., Larrainzar, E., Seminario, A., Díaz-Leal, J. L., Alamillo, J. M., and Pineda, M., et al. (2013). Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean. *J. Exp. Bot.* 64, 2171–2182. doi: 10.1093/jxb/ert074

Hao, G., Zhang, D.-X., Zhang, M.-Y., Guo, L.-X., and Li, S.-J. (2003). Phylogenetics of *Bauhinia* subgenus *Phanera* (Leguminosae: caesalpinioideae) based on ITS sequences of nuclear ribosomal DNA. *Bot. Bull. Acad. Sin.* 44, 223–228. doi: 10.1093/jxb/ert074

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal. Behav.* 7, 1456–1466. doi: 10.4161/psb.21949

Kandjimi, O. S., Uzabakiriho, J. D., and Chimwamurombe, P. M. (2015). Isolation and characterization of culturable bacteria from bulk soil samples and the rhizosphere of arid-adapted *Tylosema esculentum* (Burchell). A. Schreiber (marama bean) in Namibia. *Afr. J. Biotechnol.* 14, 944–952.

Karamanos, A. J., and Travlos, I. S. (2012). The water relations and some drought tolerance mechanisms of the marama bean. *Agron. J.* 104, 65–72. doi: 10.2134/agronj2011.0194

Keegan, A. B., and Van Staden, J. (1981). Marama bean, *Tylosema esculentum*, a plant worthy of cultivation. *S. Afr. J. Sci.* 77:387.

Kim, Y., and Cullis, C. (2017). A novel inversion in the chloroplast genome of marama (*Tylosema esculentum*). *J. Exp. Bot.* 68, 2065–2072. doi: 10.1093/jxb/erw500

King, C. A., and Purcell, L. C. (2006). Genotypic variation for shoot N concentration and response to water deficits in soybean. *Crop Sci.* 46, 2396–2402. doi: 10.2135/cropsci2006.03.0165

Lawlor, D. W. (2018). "Marama bean (*Tylosema esculentum*): a revies of morphological and physiological adaptations to environment, and crop potential," in *A Praise of Demeter. Studies in Honour of Professor A. J. Karamanos*, eds E. Eleftherohorinos, Paplomatas, and G. Economou-Antonaka (Editions Papazissi, Athens), 412.

Lewis, G., Schrire, B., MacKinder, B., and Lock, M. (2005). *Legumes of the World Royal Botanical Gardens*. Kew: Royal Botanic Gardens.

Li, T., Hu, Y. J., Hao, Z. P., Li, H., Wang, Y. S., and Chen, B. D. (2012). First cloning and characterization of two functional aquaporin genes from an arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytol.* 197, 617–630. doi: 10.1111/nph.12011

Lin, H., Chen, Y., Zhang, H., Fu, P., and Fan, Z. (2017). Stronger cooling effects of transpiration and leaf physical traits of plants from a hot dry habitat than from a hot wet habitat. *Funct. Ecol.* 31, 2202–2211. doi: 10.1111/1365-2435.12923

Mitchell, R. A. C., Keys, A. J., Madgwick, P. J., Parry, M. A. J., and Lawlor, D. W. (2005). Adaptation of photosynthesis in marama bean - *Tylosema esculentum* (Burchell a. schreiber) to a high temperature, high radiation, drought-prone environment. *Plant Physiol. Biochem.* 43, 969–976. doi: 10.1016/j.plaphy.2005.08.009

Morgan, J. A. W., Bending, G. D., and White, P. J. (2005). Biological costs and benefits to plant–microbe interactions in the rhizosphere. *J. Exp. Bot.* 56, 1729–1739. doi: 10.1093/jxb/eri205

National Research Council (1979). *Tropical Legumes: Resources for the Future.* Washington, DC: The National Academies Press, 68–74.

National Research Council (2006). *Lost Crops of Africa: Volume II: Vegetables*. Washington, DC: The National Academies Press.

Naylor, R. L., Falcon, W. P., Goodman, R. M., Jahn, M. M., Sengooba, T., Tefera, H., and Nelson, R. J. (2004). Biotechnology in the developing world: a case for increased investments in orphan crops. *Food Policy* 29, 15–44. doi: 10.1016/j.foodpol.2004.01.002

Nepolo, E., Takundwa, M., Chimwamurombe, P. M., Cullis, C. A., and Kunert, K. (2009). A review of geographical distribution of marama bean [*Tylosema esculentum* (Burchell) Schreiber] and genetic diversity in the Namibian germplasm. *Afr. J. Biotechnol.* 8, 2088–2093.

Nyembwe, P. M., de Kock, H. L., and Taylor, J. R. N. (2018). Potential of defatted marama flour-cassava starch composites to produce functional gluten-free bread-type dough. *LWT* 92, 429–434. doi: 10.1016/j.lwt.2018.02.062

Osakabe, Y, Osakabe, K., Shinozaki, K., and Tran, L.-S. P. (2014). Response of plants to water stress. *Front. Plant Sci.* 5:86. doi: 10.3389/fpls.2014.00086

Peoples, M. B., Murray, J. U., and David, H. (2009). "Measuring symbiotic nitrogen fixation by legumes," in *Nitrogen Fixation in Crop Production*, eds Emerich, D. W and Krishnan, H. B, (Madison, WI: American Society of Agronomy), 125–170.

Porcel, R., Aroca, R., Azcón, R., and Ruiz-Lozano, J. M. (2006). PIP aquaporin gene expression in arbuscular mycorrhizal Glycine max and *Lactuca sativa* in relation to drought stress tolerance. *Plant Mol. Biol.* 60, 389–404. doi: 10.1007/s11103-005-4210-y

Powell, A. M. (1987). Marama Bean (*Tylosema esculentum*, Fabaceae) seed crop in Texas. *Econ. Bot.* 41, 216–220. doi: 10.1007/BF02858969

Rakocevic, M., Matsunaga, F. T., Müller, M., Nepomuceno, A. L., Farias, J. R. B., and Neumaier, N. (2017). "Stress-induced DREB1A gene changes heliotropism and reduces drought stress in soybean plants under greenhouse conditions," in *Proceedings of the 2016 IEEE International Conference on Functional-Structural Plant Growth Modeling, Simulation, Visualization and Applications (FSPMA) Publication Year: 2016*, Qingdao, 183–188. doi: 10.1109/FSPMA.2016.7818305

Rapparini, F., and Peñuelas, J. (2013). "Mycorrhizal fungi to Alleviate drought stress on plant growth," in *Use of Microbes for the Alleviation of Soil Stresses*, Vol. 1, New York, NY: Springer Science+Business Media.

Saxon, E. C. (1981). Tuberous legumes: preliminary evaluation of tropical Australian and introduced species as fuel crops. *Econ. Bot.* 35, 163–173. doi: 10.1007/BF02858683

Serraj, R., Sinclair, T. R., and Purcell, L. C. (1999). Symbiotic N2 fixation response to drought. *J. Exp. Bot.* 50, 143–155. doi: 10.1093/jxb/50.331.143

Shavrukov, Y, Kurishbayev, A., Jatayev, S., Shvidchenko, V., Zotova, L., and Koekemoer, F., et al. (2017). Early flowering as a drought escape mechanism in plants: how can it aid wheat production? *Front. Plant Sci.* 8:1950. doi: 10.3389/fpls.2017.01950

Smith, W. (1978). Temperature of desert plants: another perspective on the adaptability of leaf size. *Science* 201, 614–616. doi: 10.1126/science.201.4356.614

Tátrai, Z. A., Sanoubar, R., Pluhár, Z., Mancarella, S., Orsini, F., and Gianquinto, G. (2016). Morphological and physiological plant responses to drought stress in *Thymus citriodorus*. *Inter. J. Agron.* 8:2016. doi: 10.1155/2016/4165750

Travlos, I. S., and Karamanos, A. J. (2008). Growth responses of marama bean (*Tylosema esculentum*) to water deficit conditions. *Inter. J. Agric. Res.* 3, 155–160. doi: 10.3923/ijar.2008.155.160

Travlos, I. S., Liakopoulos, G., Karabourniotis, G., Fasseas, C., and Karamanos, A. J. (2008). Circadian leaflet movements of *Tylosema esculentum* (Burch) A. Schreib, and the abolishment of these diurnal movements by potassium deficiency. *J. Arid Environ.* 72, 1745–1750. doi: 10.1016/j.jaridenv.2008.03.020

Trytsman, M., van Wyk, A. E., and Masemola, E. L. (2011). Systematics, diversity and forage value of indigenous legumes of South Africa, Lesotho and Swaziland. *Afr. J. Biotechnol.* 10, 13773–13779. doi: 10.5897/AJB10.2224

van Zanten, M., Pons, T. L., Janssen, J. A. M., Voesenek, L. A. C. J., and Peeters, A. J. M. (2010). On the relevance and control of leaf angle. *Critical Rev. Plant Sci.* 29, 300–316. doi: 10.1080/07352689.2010.502086

Villalobos-Rodriquez, E., and Shibles, R. (1985). Response of determinate and indeterminate tropical soybean cultivars to water stress. *Field Crops Res.* 10, 269–281. doi: 10.1016/0378-4290(85)90033-4

Weise, T., Kai, M., and Piechulla, B. (2013). Bacterial ammonia causes significant plant growth inhibition. *PLoS One* 8:e63538. doi: 10.1371/journal.pone.0063538

White, F. (1998). "The vegetative structure of African Ebenaceae and the evolution of rheophytes and ring species," in *The Biological Monograph: the Importance of Field Studies and Functional Syndromes for Taxonomy and Evolution of Tropical Plants*, eds Fortune-Hopkins, H.C., Huxley, C.R., Pannell, C.M., Prance, G.T., White, F (Royal Botanical Gardens, Kew).

Wunderlin, R. P. (2010). Reorganization of the Cercideae (Fabaceae: caesalpinioideae). *Phytoneuron* 48, 1–5.

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.

frontiers in Plant Science

MINI REVIEW
published: 22 October 2018
doi: 10.3389/fpls.2018.01524

Warm Season Grain Legume Landraces From the South of Europe for Germplasm Conservation and Genetic Improvement

Antonio M. De Ron[1], Penelope J. Bebeli[2], Valeria Negri[3], Maria Carlota Vaz Patto[4] and Pedro Revilla[1]*

[1] Department of Genetics and Plant Breeding, Misión Biológica de Galicia, National Spanish Research Council (CSIC), Pontevedra, Spain, [2] Laboratory of Plant Breeding and Biometry, Department of Crop Science, Agricultural University of Athens, Athens, Greece, [3] Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi di Perugia, Perugia, Italy, [4] Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal

Currently, there is a high concern from consumers regarding food quality, with emphasis on the origin of food sources. We here review the current situation of beans (*Phaseolus* spp.) and cowpea (*Vigna unguiculata* (L.) Walp.) landraces in the South of Europe focusing on morpho-agronomic and genetic diversity and physiological adaptation to the different agrosystems, including the symbiotic association with rhizobia. Despite the reduction in the production and consumption of grain legumes in Southern Europe, the variability of common bean, runner bean and cowpea landraces in this region is adequately preserved *ex situ* in germplasm banks and in breeder collections in Portugal, Spain, Italy and Greece; however, on-farm (*in situ*) conservation in isolated areas mainly in gardens and small fields for farmers own consumption and local markets is not guaranteed. This variability can be used for the genetic improvement of varieties, which will result in environmental-friendly improved legumes for a sustainable production in the South of Europe as well as in other regions of the World.

Keywords: adaptation, diversity, breeding, populations, *Phaseolus*, physiology, plant genetic resources, *Vigna unguiculata*

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Deidre Fourie, Agricultural Research Council of South Africa (ARC-SA), South Africa
Josefina Hernández Nistal, Universidade de Santiago de Compostela, Spain

****Correspondence:***
Antonio M. De Ron
amderon@mbg.csic.es

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *04 July 2018*
Accepted: *28 September 2018*
Published: *22 October 2018*

Citation:
De Ron AM, Bebeli PJ, Negri V, Vaz Patto MC and Revilla P (2018) Warm Season Grain Legume Landraces From the South of Europe for Germplasm Conservation and Genetic Improvement. Front. Plant Sci. 9:1524. doi: 10.3389/fpls.2018.01524

LEGUME LANDRACES

Food legumes are an important component of human diet and life for their contribution as source of protein but also for their support to the environment sustainability through the biological symbiotic fixation of nitrogen, and the enhancement of the ecosystem services because some of them are bee pollinated (De Ron, 2015; Suso et al., 2016). In the South of Europe, beans and cowpea are relevant nutritional and environmental resources well adapted to their agrosystem that should be genetically preserved and improved for their efficient use.

Landraces are traditional crop varieties or populations growing in specific locations and constitute valuable sources for breeding purposes as basic genetic material to obtain improved elite varieties. Usually a landrace is a mixture of a number of distinct homozygous lines in the case of self-pollinating crops (common bean, *Phaseolus vulgaris* L. and cowpea, *Vigna unguiculata* (L.) Walp.) (Raggi et al., 2013). In the case of cross-pollinated crops (runner or scarlet bean *P. coccineus* L.), the landraces are populations with more heterozygous components (Newton et al., 2010). They are maintained by farmers according to their preferences and the adaptation to their environment in ecological key areas. Merging several definitions (Spataro and Negri, 2013), a landrace can be

defined as a "variable population, which is identifiable and usually has a local name, lacks formal crop improvement, is characterized by a specific adaptation to the environmental conditions of the cultivation area (tolerant to the biotic and abiotic physiological stresses of that area) and is closely associated with the uses, knowledge, habits, dialects, and celebrations of the people who have developed and continue to grow it" (Negri et al., 2009). As such, they are a cultural and biological diversity heritage of value for present and future generations.

Common bean, runner or scarlet bean and cowpea are the warm season Mediterranean legumes included in this review. Common bean is the most important food legume for direct human consumption on a global scale (De Ron et al., 2016a), while runner bean has a more limited cultivation. Cowpea is extensively cultivated in tropical and subtropical areas in Africa (especially in the Sub-Saharan Africa, SSA) and the Americas (Central and South America), but has limited importance in Southern Europe and in North America (De Ron, 2015).

These legumes could be used for fresh and dry seeds and fresh pods and they play an important role in the healthy European Mediterranean diet. Recently, the role of beans and other food legumes in human diet refers not only to its high protein content but also to the functional properties of some components that could contribute to reduce risk of several serious diseases (Hangen and Bennink, 2003; Thompson et al., 2009; Vaz Patto et al., 2015). Recent trends on legume nutritional quality key factors focus on new strategies to enhance consumer acceptance and improve legume functional properties.

In spite of the decrease of grain legumes cultivation and consumption in some countries of Europe in the last years (**Figure 1**), the interest in landraces of these crops has recently grown in Europe, as well as in other continents This is due to the need of having a more sustainable agriculture, meet the present environmental challenges, avoid further genetic erosion and satisfy consumer increasing request for healthy, environmentally friendly, local food (with reduced physiological carbon footprint since it is locally produced). Special mention deserves the varieties that are recognized with some figure of legal protection, such as quality labels [like the European Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialties Guaranteed (TSG)]. However, the wide variability and the lack of uniformity for many morpho-agronomic traits in landraces is an obstacle for the application of the current legislation for their commercial or protected registration in some countries.

EUROPEAN COWPEA AND BEAN LANDRACES EVOLUTION AND DISTINCTNESS FROM THE ORIGINAL GENEPOOLS

Cowpea is the old "bean" that was grown by the Romans and Arabs (Álvarez de Morales, 2002). Domesticated in the SSA during the second millennium B.C., cowpea early spread in Asia and Europe, where it was grown by the Greeks in the third century B.C. and by the Romans in the first century based on the writings of Theophrastus and Plinius. With its spreading across the Old World, many different forms and landraces were developed also for this crop (Polegri and Negri, 2010).

Cowpea has been largely cultivated in the Old World, where this crop has a high cultural and socio-economic value for local communities (De Luca et al., 2018). Fresh pods of cowpea are consumed in Southern Europe, where a relatively large number of landraces has been developed, giving rise to a wide genotypic and phenotypic diversity among and within landraces (Lazaridi et al., 2017). These authors found that differences among cowpea landraces are not determined by the country of origin in Southern Europe. Neighbor landraces can be adequately distinguished even though there is a high level of diversity present within each landrace; consequently, the best strategy for maintaining diversity in an area is to preserve each of the landraces in the farms from which it came (Tosti and Negri, 2005).

After the initial domestication process in the Americas, the common and runner bean arrived in Spain and spread across Europe and later arrived in Africa (Gepts and Bliss, 1988; De Ron et al., 2015, 2016a). Since these species were originated and domesticated in tropical highlands, local widely different biotic and physiological abiotic conditions and farmer preferences and/or initial genetic bottleneck had a strong influence on the development of European landraces (Rodiño et al., 2006; Rodriguez et al., 2013; Raggi et al., 2014; Ferreyra et al., 2017) which resulted in a complex genetic structure of the bean germplasm and in a clear differentiation of the European gene pool with respect to the American genetic pools (Santalla et al., 2002; Angioi et al., 2010; Spataro et al., 2011; Rodriguez et al., 2013).

As for common bean in particular, no records of this crop have been found in European herbariums earlier than 1543; but, according to Zeven (1997), common bean was certainly widely grown in many areas of Europe in 1669. Of the many landraces found across Europe most belong to the Andean gene pool, being less represented the Mesoamerican gene pool (Rodiño et al., 2001; Angioi et al., 2010; Leitão et al., 2017). However, a relatively high proportion of the European common bean germplasm (33–44%) appear to be derived from hybridization events between the Andean and Mesoamerican gene pools, when the landraces were grown in proximity, displaying novel genetic combinations not typical of the primary American centers of domestication and emphasizing the potential value of the European germplasm for breeding (Santalla et al., 2002; Angioi et al., 2010).

ECO-PHYSIOLOGICAL ADAPTIVE TRAITS OF BEAN AND COWPEA IN THE SOUTH OF EUROPE

The evolution of beans and cowpea in the South of Europe by their adaptation to the ecophysiological regional conditions has involved changes in landraces. In a study that included 10 cowpea landraces from the South of Europe (five from Portugal, three from Spain, and two from Greece) cultivated in three different locations for 2 years, Martos-Fuentes et al. (2017) displayed the

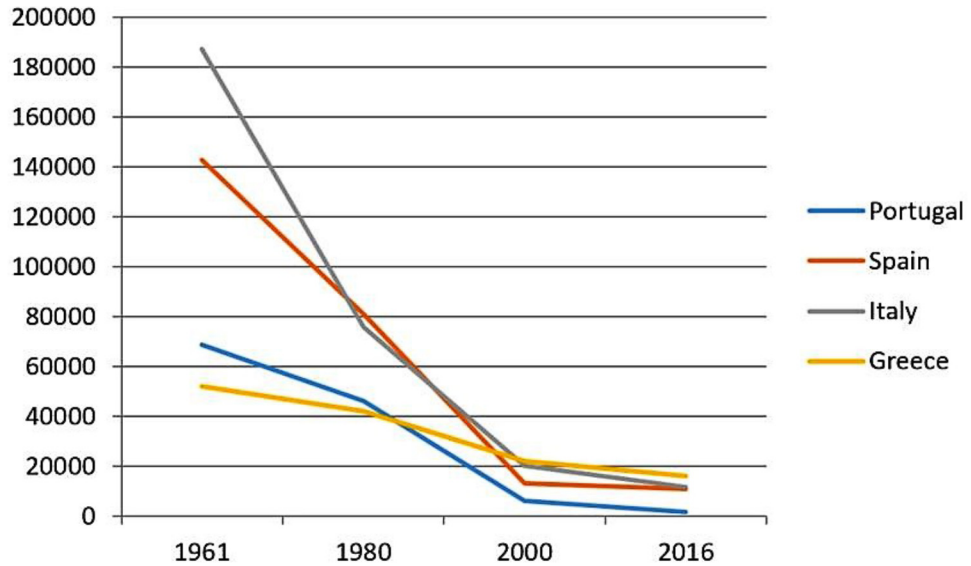

FIGURE 1 | Production (Mg/year) evolution of dry bean (*Phaseolus vulgaris*) in southern European countries (FAOSTAT, 2018).

existence of significant interactions among genotypes, locations and years, that is relevant in breeding for important agro-morpho-physiological traits.

The diversity originated in cowpea along centuries is also important for the tolerance to local stresses that each local variety has developed due to natural selection for adaptation as well as farmer selection for agronomical applications and dietary benefits (De Luca et al., 2018).

Low Temperature

A desirable characteristic for crops is a rapid and homogeneous seed germination and emergence at different environmental conditions (Revilla et al., 2005). As other crops, there are differences among bean landraces regarding their performance (germination, seedling emergence, vegetative growth, flowering, and yield) in different environments and under different temperatures. There is a need of bean germplasm with the qualities of the grain demanded by consumers to increase the success and the added value of the bean crop; and the tolerance to low temperature after sowing at germination and emergence is a key characteristic for a good development of the crop. The eco-physiological response of beans to low temperature stress has been often studied under controlled environmental conditions in glasshouse and climatic chambers but the long-term main goal of genetic improvement for low temperature tolerance is the selection of landraces under different environmental temperatures in open field.

To analyze the response to a relevant eco-physiological condition as low temperature, De Ron et al. (2016b) performed several trials with 28 dry bean genotypes (21 landraces from Spain and Portugal and seven improved varieties from Spain, France and Japan) in open field under different temperature conditions in April (low temperature: 10–14°C), May (moderate temperature: 12–17°C) and June (warm temperature: 15–22°C) in the North of Spain. The experiment was replicated in a growth chamber resembling the same environmental conditions. Three Spanish landraces (PHA399, PHA419, and PHA1058) and the improved variety Borlotto with low temperature stress-tolerance at seedling emergence, and high yield potential could be valuable genetic material for breeding programs. Seedling emergence of the large seeded landraces from Spain belonging to the Andean genetic pool was delayed compared to the small seeded landraces from the Mesoamerican one, in the controlled growth chamber and in the open field experiments, and they showed lower emergence in the open field under realistic agronomic conditions. This fact could be explained by the evolution of the common bean in the southwest of Europe, since farmers probably selected for years large seeded bean landraces due to their high market value and used to germinate the seeds in nurseries before transplanting the seedling into the open field and no breeding actions were taken by farmers and breeders to improve germination and emergence of the large-seeded Andean landraces under low temperatures in field.

The runner bean frequently requires moderate or warm temperatures for a good emergence and growth, while low temperature at sowing delays plant emergence and early growth, and can reduce establishment of the crop when an early sowing is carried out. Rodiño et al. (2007a) evaluated runner bean germplasm in a climate-chamber: 19 landraces from Spain and Portugal, four from Mexico, four from Rwanda and five commercial varieties. Best performers in emergence and first trifoliate leaf, traits related to earliness, were four Spanish and one Portuguese landraces compared to the Mexican ones that indicated a good adaptation of this genetic material to the eco-physiological conditions in the South of Europe.

Cowpea is considered a cold susceptible crop; however, cowpea is not being improved for cold tolerance. Modern breeding programs establish as key breeding objectives the

development of drought tolerant, early maturing, pest tolerant and erect type cowpea in countries where this is an important crop, such as India (Roy et al., 2016). Since several years ago, some reports have found genetic diversity for cold tolerance in cowpea, e.g., El-Kholy et al. (1997) evaluated a collection of cowpea genotypes for cold tolerance at germination and found that genotypes differed in rate for leakage of electrolytes but not in maximal percentage of germination. The effects of cold conditions on crop development differ at diverse growth stages, from germination to reproduction. Under cold conditions in the field, reducing the seed moisture content results in decreased percentage of emergence and rapid electrolyte leakage in cowpea, while deep sowing results in slow and low percentage emergence (Ismail et al., 1997).

The genetic regulation of cold tolerance in cowpea has been scarcely studied; but several genes related to cowpea response to cold conditions have been identified (Tan et al., 2016). Ismail et al. (1997) proposed that cold tolerance at early stages of the crop is due to a seed dehydrin protein and can be explained by a single gene inheritance. These authors also reported that maternal gene effects are important for the electrolyte leakage of cowpea seeds at cold temperature, and appear to restrict their contribution to cold tolerance to the beginning of plant development. Although genetic diversity for cold tolerance is limited in cowpea, breeding cowpea for cold tolerance at germination has been successful (Ismail et al., 1997). Although some sources of cold tolerance have been identified, introgression of cold tolerance in elite germplasm is a challenge because cowpea is a mainly self-pollinating crop. Several major QTLs have been identified, even though the development of mapping populations is a difficult and time-consuming task. Markers have not been actually used in breeding; nevertheless, novel techniques, such as developing transgenic plants, RNA-Seq, and reverse genetics open new opportunities for molecular breeding (Tan et al., 2016).

Water Stress

Water deficit is considered a relevant agronomic factor limiting crop productivity and is responsible for important yield reduction in many crops (Serraj et al., 2004). The severity of drought stress is always unpredictable as it depends on factors such as occurrence and distribution of rainfall, evaporative demands of the atmosphere and moisture storing capacity of the different soils.

In the common bean, the main selection criteria for drought resistance is the plant growth and the grain yield. Rodiño et al. (2007b) evaluated 21 common bean accessions (12 landraces from Spain and Portugal and nine resistant and susceptible cultivars) in two locations in the northwest of Spain to identify those genetic materials adequate for breeding for water deficit tolerance. The Drought Intensity Index (DII) was calculated as DII = 1−Xds/Xns, being Xds and Xns the average of all the accessions under drought stress (DS) and no stress (NS) conditions. Drought Susceptibility Index (DSI) for each common bean accession was calculated following these formulae: DSI = (1−Yds/Yns)/DII, where Yds and Yns are the average yields of an accession under DS and NS conditions. Five Spanish landraces (PHA432, PHA471, PHA543, PHA683, and PHA2074) had high level of drought resistance together with two cultivars (Alavesa and Linex). These results confirm that during its evolution in Europe some common bean landraces were able to adapt to different eco-physiological conditions, such as drought.

Moreover, the variability present in 23 cowpea landraces collected from Greek fields revealed potential germplasm for drought tolerance (Lazaridi et al., 2016). Cowpea is considered a legume tolerant to heat, drought and poor soils due to its capacity to fix nitrogen even under stress conditions (Carvalho et al., 2017a; Lazaridi et al., 2017). However, the diversity available for stress tolerance in southern Europe has neither been deeply studied nor used in breeding programs for stress tolerance. Shadeya et al. (2000) found limited diversity for drought tolerance in advanced breeding lines evaluated by a rapid laboratory method, but they were able to identify tolerant and susceptible genotypes that could be used for breeding. Genetic regulation of drought tolerance follows an additive – dominance model in most crops, including cowpea; being dominance and additive effects similar and important, while epistasis was rare, and narrow sense heritability was low to moderate for most traits under terminal water stress (Olajide and Ilori, 2018).

Drought effects in cowpea included reduction of plant growth, yield components, shoot and seed nutrients, and leaf water content, along with membrane instability; while increase activity of leaf antioxidant enzymes, content of leaf proline, electrolyte leakage, and shoot Si content (Merwad et al., 2018). Furthermore, leaf anatomical features are also altered by drought, being width of midvein and xylem, thickness of midvein, phloem and xylem tissues, and palisade and spongy tissues of leaf blade decreased (Merwad et al., 2018). Drought reduces plant cell water potential and turgor and raises solute concentrations. The water deficit had negative influence on mineral nutrition and metabolism decreases leaf area and alters assimilate partitioning among the organs. Physiological mechanisms of the plants for facing water stress include escape, avoidance by increasing the water uptake and reducing transpiration rate by maintaining tissue turgor by osmotic adjustment allowing the plants to preserve their vegetative growth, and resist the severe water stress by physiological mechanisms (Jones, 2004).

Biological Nitrogen Fixation

Legume biological Nitrogen (N) fixation by symbiosis with soil rhizobia provides an eco-physiological and agronomic chance to increase common bean productivity related to soil fertility and climatic conditions. Miranda and Bliss (1991) reported that many common bean landraces have low biological N fixation capacity probably due to their original domestication process as a home garden crop, with low selection pressure for the improvement of the symbiotic association with rhizobia. Rodiño et al. (2011) studied 64 common bean landraces for their capacity to establish symbiosis with rhizobia in controlled conditions and the effect of the environmental conditions on the symbiotic efficiency of them in six environments in Spain. The variation among environments for nodulation efficiency among landraces was remarkable, and five of them (PHA175, PHA508, PHA525, PHA595, and PHA652) displayed good nodulation and high yield in field.

The bean-rhizobia symbiotic system is usually affected by the water availability. Coleto et al. (2014) studied the inhibition of N fixation and ureide accumulation under water deficit in two common bean landraces and two breeding lines of contrasting drought tolerance. Their results displayed relevant genotypic differences in the drought sensitivity of biological N fixation among the landraces, and that the genetic variation is linked to ureide accumulation in the stressed leaves. In addition, two common bean landraces studied (PHA246 from Spain and PHA683 from Portugal) had better performance under DS than the tolerant breeding line used as check (Sea 5); therefore, their eco-physiological adaptation were reliable and they could be used in breeding programs designed to improve the efficiency of N biological fixation under water stress in common bean in the South of Europe.

De Ron et al. (2014) evaluated 10 common bean landraces from Spain and Portugal, together with some breeding lines tolerant to water deficit. This material was inoculated with 10 distinct strains of *Rhizobium* (eight local and two checks, *R. tropici* CIAT899 and *R. etli* CFN42) in greenhouse both under irrigation and water stress. Under water stress, five Spanish and one Portuguese landraces displayed high nodule number, high nodular biomass, a great uniformity in the caliber of their nodules, and a significant correlation with aerial biomass that is a relevant component of plant yield.

The results of these experiments showed that the common bean landraces are well adapted to their eco-physiological environments in the South of Europe and some of them are able to establish an efficient symbiosis with native rhizobia, even under water stress conditions.

Low soil fertility is a challenge for cowpea production, especially in low-input agriculture, which is the most common production system in undeveloped countries where this legume is a basic food supply. Fortunately, this crop has a great ability to synthetize N through the symbiotic interaction with rhizobia. Adaptation of cowpea includes coevolution with indigenous rhizobia associated with strains of the species *Ensifer fredii* that were able to nodulate and fix N in cowpea but not in soybean and common bean (Tampakaki et al., 2017). These authors conclude that the *Ensifer* isolates may constitute a new symbiovar for which they propose the name "aegeanense". Furthermore, symbiosis can partially explain the gains in breeding programs for agronomic performance; actually, Oruru et al. (2018) reported that modern cultivars of cowpea had higher root colonization, nodulation, and nutrients in the shoot than old cultivars and concluded that the response of mycorrhizal inoculation has been indirectly improved by selection for yield.

MORPHO-AGRONOMIC AND GENETIC TRAITS OF BEAN AND COWPEA IN THE SOUTH OF EUROPE

In the European Mediterranean basin, clearly differentiated common bean landraces exist, originated from populations firstly introduced in the Iberian Peninsula after the exploration of The Americas. A particular case is the white seed bean types from Turkey that seem to be phylogenetically distant from the rest of the European germplasm, probably due to their introduction through East Asia via the Silk Route (De La Fuente et al., 2010).

There are great differences in the preferences of the bean markets and consumers in different countries and regions related to grain size, shape, color, and cooking time, therefore these types are described as "market classes" (Voysest, 2000; Santalla et al., 2001), usually including unimproved germplasm (landraces) and some improved varieties. Breeding for commercial varieties in beans within landraces of different market classes has the goals of improve the preferred seed size, shape, color, and pattern in each area of production. As mentioned above, in the South of Europe bean landraces appear to have experienced major phenomena of evolution and adaptation, as they show clear differences between them.

In Portugal, the national common bean production still depends considerably on landraces adapted to local conditions, and fulfilling specific morphological, agronomic and nutritional farmers' preferences in mainland north and central regions, Azores and Madeira Islands (Moreira and Veloso, 2009; Vaz Patto and Araújo, 2016). Currently there are some common bean cultivars (six landraces and two conservation varieties ("Corno de Carneiro" and "Tarrestre") registered at the Portuguese National Catalog (CNV, 2017). Based on morphological and reproductive traits, considerable diversity has been described among common bean landraces from the North of Portugal (Rodiño et al., 2001) and from Madeira Island (Freitas et al., 2010). In particular, different sources of resistance and partial resistance to rust and powdery mildew have been identified in a dedicated germplasm collection screening (Leitão et al., 2013), anticipating a high potential for disease resistance breeding in the Portuguese germplasm. The genetic variation of the Portuguese common bean accessions was also characterized using RAPD and SSR molecular markers (Martins et al., 2006; Leitão et al., 2017) not detecting clear relation between the geographic distribution and the genetic distance. This absence of relation may be due to an important genetic flow resulting from the traditional seed exchange practices at local markets or among farmers. Leitão et al. (2017) also positioned the Portuguese germplasm in the worldwide diversity of common bean through a SSR-based genetic diversity study involving an enlarged collection of Portuguese landraces from all traditional bean-growing geographical areas and wild relatives and representative accessions from the Andean and Mesoamerican gene pools. Structure analysis divided the collection into three main clusters, with most of the Portuguese accessions clustering with the Andean representatives, but one third of the analyzed national landraces might represent putative hybrids between American gene pools. Some core collections were developed by the same authors maximizing the genetic and morphological diversity of the original collection, and representing the Portuguese common bean accessions with the minimum redundancy.

In Spain many common bean landraces have been collected in farmer fields, starting from the 70's of the last century and are conserved *ex situ* in the national gene bank (CRF-INIA, Alcalá de Henares) as well as in breeders collections in different regions, while many landraces can still be found

cultivated *in situ* (on-farm) in some places mainly for own or local markets consumption. Six areas of Spain have landraces or local varieties awarded with quality labels (PDO and PGI), including 16 common bean and one runner bean, while no cowpea variety is recognized with these labels. The most appreciated landraces or local varieties are white large and extra-large seeded (50–100 g 100 seeds^{-1}) (Santalla et al., 2005), generally belonging to the Andean gene pool, although some of them are intermediate or recombinant types with the Mesoamerican pool (Santalla et al., 2002).

In Italy over 200 common bean landraces are officially recorded as maintained *in situ* (Negri et al., 2013) with six of them awarded with PDO or PGI. An analysis of 146 Italian landraces based on the combined use of morpho-physiological, biochemical and molecular traits clearly distinguished almost each landrace from the others (Raggi et al., 2013). It also showed that the Italian landrace genetic diversity is clearly structured in three clusters and that clustering is not simply ascribable to the original Mesoamerican and Andean gene pools, similarly to what was found in the Portuguese germplasm by Leitão et al. (2017). On the contrary, the distinction of cluster 1 from cluster 2 appeared to be (at least partially) due to adaptation (for flowering date and resistance/tolerance to diseases) to the different environmental conditions that are determined by altitude since the presence of selective effects was detected for some of the SSR used in the study. Breeding activities have been intense in the past years since beans are largely cultivated for both the seed and the pod consumption in Italy: twenty-eight cultivars have been released by a Ministry of Agriculture Research Center (CREA_CI, A. Carboni pers. comm.). Most of them were specifically bred for resistance to the main biotic stress of the crop (which are striking on intensive cultivations) and mostly relying on alien germplasm since landraces, although giving product of high organoleptic quality, are poorly adapted to intensive cultivation (Parisi and Campion, 2010). However, to breed lines specifically suited to organic agriculture we can well take advantage of landrace germplasm (Caproni et al., 2018).

In Greece bean landraces have been collected in organized expeditions particularly during the previous century and are conserved *ex situ* in many genebanks while several landraces can still be found cultivated on-farm in many places mainly for own or local consumption. The dry beans of several Greek *Phaseolus* spp. landraces have been characterized as PDO or PGI having added value that resulted in the need for testing the authenticity of their products and the development of test methods based on molecular tools (Ganopoulos et al., 2012). Improved cultivars bred in Greece are either selections from Greek landraces or selections following crosses between landraces and introduced germplasm. Characterization and evaluation of common bean landraces and main improved varieties cultivated in Greece using morpho-agronomical, physicochemical traits, sensory and molecular data showed a wide (among and within landraces) genetic variation and also revealed promising landraces with superior yield components and protein content that could be used "*per se*" or in breeding programs for conventional or low input/organic cultivation (Mavromatis et al., 2010; Vakali et al., 2017).

As for runner beans, different landraces are also cultivated in Spain, Portugal, and Italy (Santalla et al., 2004; Spataro et al., 2011; Rodriguez et al., 2013; Schwember et al., 2017), and in the North of Greece where two groups can be distinguished depending on seed dimensions ("giants" with 100 seed weight range from 120 to 180 g and "elephants" with 100 seed weight outreaches the 180 g).

Cowpea is currently a crop of minor importance for Europe; however, considering its greater drought resistance in comparison with common bean and a scenario of climate change and unpredictability, it is likely to have an increased

TABLE 1 | Warm season legume landraces *ex situ* collections in the South of Europe.

Country	National collections (gene banks)	Breeder collections and features
Portugal	Portuguese Plant Germplasm Bank, BPGV (Braga, Portugal, FAO code PRT001; now conserving also the previous INIAV Research Unit of Biotechnology and Genetic Resources collection, FAO code PRT005). Conserving 3307 common bean and 344 cowpea accessions.	
Spain	National Center for Plant Generic Resources (CRF-INIA, FAO code ESP004). Conserving 3616 Common bean, 121 runner bean and 487 cowpea accessions.	MBG-CSIC (FAO code ESP009) (De Ron et al., 2018). Conserving 1701 Spanish and other origins common bean, 49 Spanish runner bean and 89 Spanish and Portuguese cowpea accessions (De Ron et al., 2003).
Italy	UNIPG-DSA3 (FAO code ITA363) and other Italian collections (See https://www.crea.gov.it/853/plantares/ for details on number of conserved accessions).	Common bean 552, runner bean 91 and cowpea 16 accessions.
Greece	Greek Genebank (FAO code GRC005). Conserving 436 common bean, 30 runner bean 30 and 37 cowpea accessions (Katsiotis et al., 2009).	

importance in future years. For instance, Portugal has presently one cowpea cultivar ("Fradel") registered at the National Catalog for commercial use (CNV, 2017) although many varieties are available commercially. Cowpea cultivation is mostly based on landraces and scientific studies have been carried out to assess breeding potentialities of local germplasm (Negri et al., 2000, 2001; Lazaridi et al., 2016, 2017; Carvalho et al., 2017a,b; Karapanos et al., 2017; Martos-Fuentes et al., 2017). The characterization of fresh pod traits in thirty-one cowpea landraces from Portugal, Spain and Greece revealed promising variation for production (Lazaridi et al., 2017).

From all the above, we may conclude that Southern Europe is still rich in landrace diversity maintained *ex situ* and *in situ* which represents an important source of interesting plant traits combinations, not yet fully explored in formal genetic improvement programs. According to the PGR Genesys (2018) database the landraces of warm season legumes landraces from the South of Europe maintained in genebanks are: 11371 of common bean, 1442 of runner bean and 940 of cowpea. *In situ* wealth of landrace diversity is presently threatened by the replacement by novel, genetically uniform cultivars, the possible general effects of climate change in plant physiology and growth, the aging of farmers and ineffective transmission of knowledge related to landraces, the desertion of the land caused by migration from rural areas to cities, the internationalization of food systems and the pressure of changing markets with restrictive food standards.

However, it should be noted that some of the numerous landraces of warm season legumes were/are being awarded of EU quality marks (27, including both common and runner bean) and/or are promoted as typical product locally. This helps, at least partially, halting the loss of useful germplasm and its evolution *in situ*. The on farm conservation of landraces could be guaranteed if it offers an income to the farmers. This can be achieved by marketing the landraces products emphasizing their uniqueness with special brand names that highlight their local cultural heritage (Karanikolas et al., 2017). Additionally, *in situ* conservation can be accomplished by supporting farmers willing to cultivate the traditional varieties, for example with a participatory plant breeding program, or even with financial support when the genetic resources are considered as national patrimony.

CONCLUDING REMARKS

According to the available data, the variability of the common bean, runner bean and cowpea landraces from the South of Europe is adequately preserved *ex situ* in germplasm banks and in breeders collections in Portugal, Spain, Italy and Greece (**Table 1**); however, on-farm or *in situ* conservation in isolated areas mainly in gardens and small fields for farmers own consumption and local markets is not guaranteed currently. In addition, this variability is being used for the genetic improvement of varieties, some of them already registered and others protected by quality labels, despite the reduction in the production and consumption of grain legumes in those areas. Legume research programs in Europe are only focussed to cowpea pre-breeding, even though this crop could make significant contributions to legume production in arid areas.

The genetic structure of landrace populations, in the case of autogamous species such as common bean and cowpea, gives an opportunity for individual selection within landraces adapted to particular eco-physiological conditions with the objective of obtaining improved breeding lines that could be used *per se* for production or as basic germplasm for breeding programs. In the case of the runner bean, an allogamous species, individual selection must include isolation because of the role of insects in the reproductive process.

To take full advantage of these valuable bean and cowpea adapted landrace resources it is extremely important to complement the existing molecular/morpho-physiological diversity analysis with detailed phenotypic evaluations, and to enhance the symbiotic system legume-rhizobia for an efficient biological N fixation. These will allow the identification of landraces with increased market value (adapted to biotic and physiological stresses or characterized by market quality traits) that can actively be used to overcome different constraints affecting both production and consumption. It will result in obtaining environmental-friendly improved legumes for a sustainable production in the South of Europe and for other regions of the World.

AUTHOR CONTRIBUTIONS

ADR, PB, VN, MVP, and PR equally contributed to the conception of the work, revising the work, and approval of the version to be published.

FUNDING

Research was supported by the projects RFP2013-00001 and RFP105-0008 from the Spanish Government and AGI/CSICI + D + I. OTR00114 from the Galician Government – CSIC (Spain), IF/01337/2014 and UID/Multi/04551/2013 from Fundação para a Ciência e Tecnologia (Portugal). ADR and PR acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

ACKNOWLEDGMENTS

The authors are grateful to CRF-INIA (Alcalá de Henares, Spain), Polytechnic University of Cataluña (Barcelona, Spain), and SERIDA (Villaviciosa, Spain) for supplying common bean runner bean and cowpea germplasm, and Diputación de Pontevedra (Pontevedra, Spain) for farm facilities.

REFERENCES

Álvarez de Morales, C. (2002). "Agrónomos andalusíes y sus legados," in *La Herencia Árabe en la Agricultura y el Bienestar de Occidente*, ed. F. Nuez (Valencia: Editorial Universidad Politécnica de Valencia), 1–69.

Angioi, S., Rau, D., Attene, G., Nanni, L., Bellucci, E., Logozzo, G., et al. (2010). Beans in Europe: origin and structure of the European landraces of *Phaseolus vulgaris* L. *Theor. App. Genet.* 121, 829–843. doi: 10.1007/s00122-010-1353-2

Caproni, L., Raggi, L., Tissi, C., Howlett, S., Torricelli, R., and Negri, V. (2018). Multi-environment evaluation and genetic characterisation of common bean breeding lines for organic farming systems. *Sustainability* 10, 1–17. doi: 10.3390/su10030777

Carvalho, M., Bebeli, P. J., Pereira, G., Casgro, I., Egea-Gilabert, C., Matos, M., et al. (2017a). European cowpea landraces for a more sustainable agriculture system and novel foods. *J. Sci. Food Agric.* 97, 4399–4407. doi: 10.1002/jsfa.8378

Carvalho, M., Muñoz-Amatriaín, M., Castro, I., Lino-Neto, T., Matos, M., Egea-Cortines, M., et al. (2017b). Genetic diversity and structure of Iberian Peninsula cowpeas compared to world-wide cowpea accessions using high density SNP markers. *BMC Genomic* 18:891. doi: 10.1186/s12864-017-4295-0

CNV (2017). *Catálogo Nacional de Variedades. Direcção Geral da Alimentação e Veterinária (DGAV). Ministério da Agricultura, Florestas e Desenvolvimento Rural. Portugal*. Available at: www.drapc.min-agricultura.pt/base/documentos/cnv_2017.pdf [accessed May 23, 2018].

Coleto, I., Pineda, M., Rodiño, A. P., De Ron, A. M., and Alamillo, J. (2014). Comparison of inhibition of N2 fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance. *Ann. Bot.* 113, 1071–1082. doi: 10.1093/aob/mcu029

De La Fuente, M., De Ron, A. M., Rodiño, A. P., and Santalla, M. (2010). Evolution of the European bean from their American ancestors. *Annu. Rept. Bean Improv. Coop.* 53, 34–35.

De Luca, D., Cennamo, P., Del Guacchio, E., Di Novella, R., and Caputo, P. (2018). Conservation and genetic characterisation of common bean landraces from Cilento region (southern Italy): high differentiation in spite of low genetic diversity. *Genetica* 146, 29–44. doi: 10.1007/s10709-017-9994-6

De Ron, A. M. (2015). *Grain Legumes. Series: Handbook of Plant Breeding*. New York: Springer Science + Business Media, doi: 10.1007/978-1-4939-2797-5

De Ron, A. M., De la Rosa, L., and Marcos, T. (2018). Current situation of the bean germplasm collections in Spain. *Annu. Rept. Bean Improv. Coop.* 61, 53–54.

De Ron, A. M., González, A. M., Monteagudo, A. B., Lema, M., and Santalla, M. (2003). Colección de Vigna spp. de la MBG-CSIC. *Act. Assoc. Esp. Legum.* 2, 121–123.

De Ron, A. M., González, A. M., Rodiño, A. P., Santalla, M., Godoy, L., and Papa, R. (2016a). History of the common bean crop: its evolution beyond its areas of origin and domestication. *Arbor* 192:a317. doi: 10.3989/arbor.2016.779n3007

De Ron, A. M., Rodiño, A. P., Santalla, M., González, A. M., Lema, M. J., Martín, I., et al. (2016b). Seedling emergence and phenotypic response of common bean germplasm to different temperatures under controlled conditions and in open field. *Front. Plant Sci.* 7:1087. doi: 10.3389/fpls.2016.01087

De Ron, A. M., Papa, R., Bitocchi, E., González, A. M., Debouck, D. G., Brick, M. A., et al. (2015). "Common Bean," in *Grain Legumes. Series: Handbook of Plant Breeding*, ed. A. M. De Ron (New York, NY: Springer Science + Business Media), 1–36. doi: 10.1007/978-1-4939-2797-5-1

De Ron, A. M., Rodiño, A. P., and Riveiro, M. (2014). Analysis of the symbiotic system bean-rhizobia under water stress conditions in greenhouse. *Annu. Rept. Bean Improv. Coop.* 57, 99–100.

El-Kholy, A. S., Hall, A. E., and Mohsen, A. A. (1997). Heat and chilling tolerance during germination and heat tolerance during flowering are not associated in cowpea. *Crop Sci.* 37, 456–463. doi: 10.2135/cropsci1997.0011183X003700020025x

FAOSTAT (2018). Available at: http://www.fao.org/faostat/en/#data/QC [accessed May 12, 2018].

Ferreyra, M., Menéndez-Sevillano, M. C., Ibarra, L. R., and De Ron, A. M. (2017). Characterization of wild common bean populations for their in situ conservation in Northwestern Argentina. *Euphytica* 213:186. doi: 10.1007/s10681-017-1954-9

Freitas, G., Ganança, J. F. T., Nóbrega, H., Nunes, E., Costa, G., Slaski, J. J., et al. (2010). Morphological evaluation of common bean diversity on the Island of Madeira. *Genet. Resour. Crop Evol.* 58, 861–874. doi: 10.1007/s10722-010-9624-y

Ganopoulos, I., Bosmali, I., Madesis, P., and Tsaftaris, A. (2012).). Microsatellite genotyping with HRM (High Resolution Melting) analysis for identification of the PGI common bean variety Plake Megalosperma Prespon. *Eur. Food Res. Technol.* 234, 501–508. doi: 10.1007/s00217-011-1653-z

Gepts, P., and Bliss, F. A. (1988). Dissemination pathways of common bean (*Phaseolus vulgaris*, Fabaceae) deduced from phaseolin electrophoretic variability. II. Europe and Africa. *Econ. Bot.* 42, 86–104. doi: 10.1007/BF02859038

Hangen, L., and Bennink, M. R. (2003). Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced azoxymethane-induced colon cancer in rats. *Nutr. Cancer* 44, 60–65.

Ismail, A. M., Hall, A. E., and Close, T. J. (1997). Chilling tolerance during emergence of cowpea associated with a dehydrin and slow electrolyte leakage. *Crop Sci.* 37, 1270–1277. doi: 10.2135/cropsci1997.0011183X003700040041x

Jones, H. (2004). "What is water use efficiency?," in *Water Use Efficiency in Plant Biology*, ed. M. A. Bacon (Oxford: Blackwell), 27–41.

Karanikolas, P., Bebeli, P. J., and Thanopoulos, R. (2017). Farm economic sustainability and agrobiodiversity: identifying viable farming alternatives during the economic crisis in Greece. *J. Environ. Econ. Policy* 7, 69–84. doi: 10.1080/21606544.2017.1360212

Karapanos, I., Papandreou, A., Skouloudi, M., Makrogianni, D., Fernández, J. A., Rosa, E., et al. (2017). Cowpea fresh pods - a new legume for the market: assessment of their quality and dietary characteristics of 37 cowpea accessions grown in southern Europe. *J. Sci. Food Agric.* 97, 4343–4352. doi: 10.1002/jsfa.8418

Katsiotis, A., Mavromatis, A., Bebeli, P., and Tzivelikas, A. (2009). "An inventory of Greek landraces," in *European Landraces: On-farm Conservation, Management and Use*, eds M. Vetelainen, V. Negri, and N. Maxted (Rome: Bioversity Int. Publications), 97–104.

Lazaridi, E., Ntatsi, G., Fernández, J. A., Karapanos, I., Carnide, V. P., Savvas, D., et al. (2017). Phenotypic diversity and evaluation of fresh pods of Southern European cowpea (*Vigna unguiculata* (L.) Walp.) landraces. *J. Sci. Food Agric.* 97, 4265–4655. doi: 10.1002/jsfa.8249

Lazaridi, E., Ntatsi, G., Savvas, D., and Bebeli, P. J. (2016). Diversity in cowpea (*Vigna unguiculata* (L.) Walp.) local populations from Greece. *Genet. Resour. Crop Evol.* 64, 1529–1551. doi: 10.1007/s10722-016-0452-6

Leitão, S. T., Almeida, N. F., Moral, A., Rubiales, D., and Vaz Patto, M. C. (2013). Identification of resistance to rust (*Uromyces appendiculatus*) and powdery mildew (Erysiphe diffusa) in Portuguese common bean germplasm. *Plant Breed.* 132, 654–657. doi: 10.1111/pbr.12094

Leitão, S. T., Dinis, M., Veloso, M. M., Satovic, Z., and Vaz Patto, M. C. (2017). Establishing the bases for introducing the unexplored Portuguese common bean germplasm into the breeding world. *Front. Plant Sci.* 8:1296. doi: 10.3389/fpls.2017.01296

Martins, S. R., Vences, F. J., Sáenz de Miera, L. E., Barroso, M. R., and Carnide, V. (2006). RAPD analysis of genetic diversity among and within Portuguese landraces of common white bean (*Phaseolus vulgaris* L.). *Sci. Hort.* 108, 133–142. doi: 10.1016/j.scienta.2006.01.031

Martos-Fuentes, M., Fernández, J. A., Ochoa, J., Carvalho, M., Carnide, V., Rosa, E., et al. (2017). Genotype by environment interactions in cowpea (*Vigna unguiculata* L. Walp.) grown in the Iberian Peninsula. *Crop Pasture Sci.* 68, 924–931. doi: 10.1071/CP17071

Mavromatis, A. G., Arvanitoyannis, I. S., Korkovelos, A. E., Giakountis, A., Chatzitheodorou, V. A., and Goulas, C. K. (2010). Genetic diversity among common bean (*Phaseolus vulgaris* L.) Greek landraces and commercial cultivars: nutritional components, RAPD and morphological markers. *Spanish J. Agric. Res.* 8, 986–994. doi: 10.5424/sjar/2010084-1245

Merwad, A. R. M. A., Desoky, E. S. M., and Rady, M. M. (2018). Response of water deficit-stressed *Vigna unguiculata* performances to silicon, proline or

methionine foliar application. *Sci. Hort.* 228, 132–144. doi: 10.1016/j.scienta.2017.10.008

Miranda, B. D., and Bliss, F. A. (1991). Selection for increased seed nitrogen accumulation in common bean: implications for improving dinitrogen fixation and seed yield. *Plant Breed.* 106, 301–311. doi: 10.1111/j.1439-0523.1991.tb00515.x

Moreira, P. M. R. M., and Veloso, M. M. (2009). "Landrace inventory for Portugal," in *European Landraces On-farm Conservation, Management and Use*, Vol. 15, eds M. Vetelainen, V. Negri, and N. Maxted (Rome: Bioversity International), 124–136.

Negri, V., Floridi, S., and Montanari, L. (2001). Organoleptic and chemical evaluation of Italian cowpea (*Vigna unguiculata* subsp. unguiculata (L) Walp.) landraces from a restricted area. *Italian J. Food Sci.* 13, 383–390.

Negri, V., Maxted, N., and Vetelainen, M. (2009). European landrace conservation: an introduction. *Bioversity Techn. Bull.* 15, 1–22.

Negri, V., Pacicco, L., Bodesmo, M., and Torricelli, R. (2013). *The First Italian Inventory of in Situ Maintained Landraces. On CD ROM. ISBN 978-88-6074-279-7*. Perugia: Morlacchi Editrice.

Negri, V., Tosti, N., Falcinelli, M., and Veronesi, F. (2000). Characterization of thirteen cowpea landraces from Umbria (Italy) Strategy for their conservation and promotion. *Genet. Resour. Crop Evol.* 47, 141–146. doi: 10.1023/A:1008714108583

Newton, A. C., Akar, T., Baresel, J. P., Bebeli, P. J., Bettencourt, E., Bladenopoulos, K. V., et al. (2010). Cereal landraces: an essential resource for the future of contemporary agriculture, A review. *Agron. Sustain. Dev.* 30, 237–269. doi: 10.1051/agro/2009032

Olajide, A. A., and Ilori, C. O. (2018). Genetic analysis of seedlings characters associated with drought tolerance in cowpea under a controlled environment. *Plant Gen. Res. Charac. Util.* 16, 218–227. doi: 10.1017/S1479262117000235

Oruru, M. B., Njeru, E. M., Pasquet, R., and Runo, S. (2018). Response of a wild-type and modern cowpea cultivars to arbuscular mycorrhizal inoculation in sterilized and non-sterilized soil. *J. Plant Nutr.* 41, 90–101. doi: 10.1080/01904167.2017.1381728

Parisi, B., and Campion, B. (eds). (2010). *Il Fagiolo: Consiglio per la Ricerca e la Sperimentazione in Agricoltura Edizioni*. Montanaso Lombardo: Cartaceo.

PGR Genesys (2018). Available at: www.genesys-pgr.org [accessed May 28, 2018].

Polegri, L., and Negri, V. (2010). Molecular markers for promoting agro-biodiversity conservation: a case study from Italy. How cowpea landraces were saved from extinction. *Genet. Resour. Crop Evol.* 57, 867–880. doi: 10.1007/s10722-009-9526-z

Raggi, L., Tiranti, B., and Negri, V. (2013). Italian common bean landraces: diversity and population structure. *Genet. Resour. Crop Evol.* 60, 1515–1530. doi: 10.1007/s10722-012-9939-y

Raggi, L., Tissi, C., Mazzucato, A., and Negri, V. (2014). Molecular polymorphism related to flowering trait variation in a *Phaseolus vulgaris* L. collection. *Plant Sci.* 21, 180–189. doi: 10.1016/j.plantsci.2013.11.001

Revilla, P., Butrón, A., Cartea, M. E., Malvar, R. A., and Ordás, A. (2005). "Breeding for cold tolerance," in *Abiotic Stresses: Plant Resistance Through Breeding and Molecular Approaches*, eds M. Ashraf and P. J. C. Harris (New York, NY: The Harworth Press), 301–398.

Rodiño, A. P., De La Fuente, M., De Ron, A. M., Lema, M. J., Drevon, J. J., and Santalla, M. (2011). Variation for nodulation and plant yield of common bean genotypes and environmental effects on the genotype expression. *Plant Soil* 346, 349–361. doi: 10.1007/s11104-011-0823-x

Rodiño, A. P., Lema, M., Pérez-Barbeito, M., Santalla, M., and De Ron, A. M. (2007a). Assessment of runner bean (*Phaseolus coccineus* L.) germplasm for tolerance to low temperature during early seedling growth. *Euphytica* 155, 63–70. doi: 10.1007/s10681-006-9301-6

Rodiño, A. P., Riveiro, M., Santalla, M., and De Ron, A. M. (2007b). Sources of variation of common bean for drought tolerance. *Annu. Rept. Bean Improv. Coop.* 50, 163–164.

Rodiño, A. P., Santalla, M., González, A. M., De Ron, A. M., and Singh, S. P. (2006). Novel genetic variation in common bean from the Iberian Peninsula. *Crop Sci.* 46, 2540–2546. doi: 10.2135/cropsci2006.02.0104

Rodiño, A. P., Santalla, M., Montero, I., Casquero, P. A., and De Ron, A. M. (2001). Diversity in common bean germplasm (*Phaseolus vulgaris* L.) from Portugal. *Genet. Resour. Crop Evol.* 48, 409–417. doi: 10.1023/a:1012248002436

Rodriguez, M., Rau, D., Angioi, S., Bellucci, E., Bitocchi, E., Nanni, L., et al. (2013). The European *Phaseolus coccineus* L. landraces: population structure and adaptation, as revealed by of cpSSRs, nuSSRs and phenotypic analyses. *PLoS One* 8:e57337. doi: 10.1371/journal.pone.0057337

Roy, A. K., Malaviyal, D. R., and Kaushal, P. (2016). Genetic improvement of fodder legumes especially dual purpose pulses. *Indian J. Genet. Plant Breed.* 76, 608–625. doi: 10.5958/0975-6906.2016.00076.6

Santalla, M., De Ron, A. M., and Voysest, O. (2001). "European bean market classes," in *Catalogue of Bean Genetic Resources*, eds M. Amurrio, M. Santalla, and A. M. De Ron (Pontevedra: Fundación Pedro Barrié de la Maza), 77–94.

Santalla, M., González, A. M., Lema, M., Rodiño, A. P., Monteagudo, A. B., and De Ron, A. M. (2005). Improvement of large-seeded common bean cultivars under sustainable cropping systems in Spain. *Euphytica* 142, 85–95. doi: 10.1007/s10681-005-0816-z

Santalla, M., Monteagudo, A. M., González, A. M., and De Ron, A. M. (2004). Agronomical and quality traits of runner bean germplasm and implications for breeding. *Euphytica* 135, 205–215. doi: 10.1023/B:EUPH.0000014912.07993.e7

Santalla, M., Rodiño, A. P., and De Ron, A. M. (2002). Allozyme evidence supporting southwestern Europe as a secondary center of genetic diversity for common bean. *Theor. Appl. Genet.* 104, 934–944. doi: 10.1007/s00122-001-0844-6

Schwember, A. R., Carrasco, B., and Gepts, P. (2017). Unraveling agronomic and genetic aspects of runner bean (*Phaseolus coccineus* L.). *Field Crops Res.* 206, 86–94. doi: 10.1016/j.fcr.2017.02.020

Serraj, R., Krishnamurthy, L., Kashiwagi, J., Kumar, J., Chandra, S., and Crouch, J. H. (2004). Variation in root traits of chickpea (*Cicer arietinum* L.) grown under terminal drought. *Field Crops Res.* 88, 115–127. doi: 10.1016/j.fcr.2003.12.001

Shadeya, L., Akundabweni, M., Monti, L., and Fonzo, D. (2000). Detached leaf test as a rapid stress appraisal in cowpea germplasm, *Vigna unguiculuta* ((L) Walp) for drought resistance. *Disc. Innovat.* 12, 132–136. doi: 10.4314/dai.v12i3.15578

Spataro, G., and Negri, V. (2013). The European seed legislation on conservation varieties: focus, implementation, present and future impact on landrace on farm conservation. *Genet. Resour. Crop Evol.* 60, 2421–2430. doi: 10.1007/s10722-013-0009-x

Spataro, G., Tiranti, B., Arcaleni, P., Bellucci, E., Attene, G., Papa, R., et al. (2011). Genetic diversity and structure of a worldwide collection of *Phaseolus coccineus* L. *Theor. Appl. Genet.* 122, 1281–1291. doi: 10.1007/s00122-011-1530-y

Suso, M. J., Bebeli, P. J., Christmann, S., Mateus, C., Negri, V., Pinheiro de Carvalho, M., et al. (2016). Enhancing legume ecosystem services through an understanding of plant-pollinator interplay. *Front. Plant Sci.* 7:333. doi: 10.3389/fpls.2016.00333

Tampakaki, A., Fotiadis, C. T., Ntatsi, G., and Savvas, D. (2017). A novel symbiovar (aegeanense) of the genus Ensifer nodulates *Vigna unguiculata*. *J. Sci. Food Agric.* 97, 4314–4325. doi: 10.1002/jsfa.8281

Tan, H. Q., Huang, H. T., Tie, M., Tang, Y., Lai, Y., and Li, H. (2016). Transcriptome profiling of two asparagus bean (*Vigna unguiculata* subsp. sesquipedalis) cultivars differing in chilling tolerance under cold stress. *Plos One* 11:e0151105. doi: 10.1371/journal.pone.0151105

Thompson, M. D., Brick, M. A., McGinley, J. N., and Thompson, H. J. (2009). Chemical composition and mammary cancer inhibitory activity of dry bean. *Crop Sci.* 49, 179–186. doi: 10.2135/cropsci2008.04.0218

Tosti, N., and Negri, V. (2005). On-going on-farm microevolutionary processes in neighbouring cowpea landraces revealed by molecular markers. *Theor. Appl. Genet.* 110, 1275–1283. doi: 10.1007/s00122-005-1964-1

Vakali, C., Baxevanos, D., Vlachostergios, D., Tamoutsidis, E., Papathanasiou, F., and Papadopoulos, I. (2017). Genetic characterization of agronomic, physiochemical, and quality parameters of dry bean landraces under low-input farming. *J. Agr. Sci. Tech.* 19, 957–967.

Vaz Patto, M. C., Amarowicz, R., Aryee, A., Boye, J., Chung, H. J., Martín-Cabrejas, M. A., et al. (2015). Achievements and challenges in improving the nutritional quality of food legumes. *Crit. Rev. Plant Sci.* 34, 105–143. doi: 10.1080/07352689.2014.897907

Vaz Patto, M. C., and Araújo, S. S. (2016). Positioning Portugal into the context of world production and research in grain legumes. *Rev. Ciências Agrárias* 39, 3–21. doi: 10.19084/RCA16161

Voysest, O. (2000). *Mejoramiento Genético Del Frijol (Phaseolus vulgaris L.). Legado de Variedades de América Latina 1930-1999*. Cali: CIAT.

Zeven, A. C. (1997). The introduction of the common bean (*Phaseolus vulgaris* L.) into Western Europe and the phenotypic variation of dry beans collected in The Netherlands in 1946. *Euphytica* 94, 319–328. doi: 10.1023/A:1002940220241

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 October 2018
doi: 10.3389/fpls.2018.01440

Recovery of a Common Bean Landrace (*Phaseolus vulgaris* L.) for Commercial Purposes

Cristina Mallor[1], Miguel Barberán[2] and Joaquín Aibar[2]*

[1] Unidad de Hortofruticultura, Centro de Investigación y Tecnología Agroalimentaria de Aragón, IA2 Instituto Agroalimentario de Aragón (CITA – Universidad de Zaragoza), Zaragoza, Spain, [2] Escuela Politécnica Superior de Huesca, IA2 Instituto Agroalimentario de Aragón (CITA – Universidad de Zaragoza), Zaragoza, Spain

The "Caparrona" bean is a landrace that was grown largely in Monzón, and for that reason, it is also known by the name of "Caparrona de Monzón." Historical references mention that in the thirties of the last century, Caparrona beans reached a production higher than 200,000 kg. Nevertheless, the increasing modernization of agriculture at the end of the 20th century enhanced its replacement by newer varieties. As a result, only a few local growers continued producing Caparrona beans mainly for family use. However, in recent years, the high demand for local products, grown with environmentally friendly farming techniques, has reawakened interest in this local bean. In order to recover the Caparrona bean crop, a study was conducted with the aim of assessing this landrace, along with all the processes, from collecting seeds to securing the *in situ* and *ex situ* conservation. Six bean samples were initially collected from local farmers and the traditional knowledge was also recorded. After the first seed-borne virus test, two samples were rejected because of the positive results for Bean Common Mosaic Virus (BCMV). The four remaining samples were evaluated in a randomized complete block design with three replications at two locations. All through the growth phase of the plants, samples were taken for a virus test. Two samples tested positive for BCMV and were discarded. Between the two healthy seed samples, regarding morphology, chemical composition, and agronomic data, no significant statistical differences were found. Therefore, both samples were selected for commercial production. The seeds obtained from the assays were transferred to a recently created producers' association, which registered a private label to commercialize the Caparrona beans as a gourmet product. Seeds are also available from the Spanish BGHZ-CITA public genebank.

Keywords: local varieties, biodiversity, BCMV, germplasm, genebank

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Francesco Di Gioia, Pennsylvania State University, United States
María Serrano, Universidad Miguel Hernández de Elche, Spain

****Correspondence:***
Cristina Mallor
cmallor@cita-aragon.es

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *30 May 2018*
Accepted: *10 September 2018*
Published: *25 October 2018*

Citation:
Mallor C, Barberán M and Aibar J (2018) Recovery of a Common Bean Landrace (Phaseolus vulgaris L.) for Commercial Purposes. Front. Plant Sci. 9:1440. doi: 10.3389/fpls.2018.01440

INTRODUCTION

The vegetable sector plays an important role in the European Union (EU), accounting for 13.7% of EU agricultural output. In 2016, the total production of vegetables in the EU was 63.9 million tons. Spain (24.1%) and Italy (17.4%) were the most important producers (Eurostat Statistic Explained, 2017). In the past, the Spanish vegetable production was characterized by a rich variety of landraces, created by farmers themselves through repeated simple selection procedures, from generation to generation. Unfortunately, this *in situ* biodiversity has been eroded due to intensification of food production and globalization, and, currently, only a few crop varieties are being commercialized, while many local varieties are neglected or underutilized (Barbieri et al., 2014).

However, nowadays, the trend is changing, and many consumers are demanding local food products for economic reasons (increase in farmers' income, greater added value for local stakeholders, etc.); social benefits (i.e., maintenance of the population in the territory); environmental concerns (decrease in transport and gas emissions, landscape conservation, and biodiversity, etc.); and because local products are perceived fresher or of better quality (Pearson et al., 2011; Richards et al., 2017). The increased consumer demand for diversity in vegetables opens up new avenues for restoring these neglected local varieties (Kreutzmann et al., 2007). In this work, we are interested in a local bean landrace, which was cultivated some years ago, but it is no longer in commercial production.

The common bean is a valuable legume for human consumption worldwide, being an important source of high-quality proteins, carbohydrates, vitamins, minerals, dietary fiber, phytonutrients, and antioxidants (Cardador-Martínez et al., 2002; Reynoso-Camacho et al., 2006). The common bean was introduced into Europe in the early decades of the 16th century from two domestic centers, the Mesoamerican and the Andean (Lioi and Piergiovanni, 2015). The Iberian Peninsula was an expansion zone and a secondary center of diversity for the common bean, generating a wealth of landraces (Santalla et al., 2002).

Among the Spanish common bean landraces, the "Caparrona" bean was grown largely in the locality of Monzón. For that reason, it is also known by the name "Caparrona de Monzón" (Raluy, 1982). Historical references mention that in the thirties of the last century, a great number of farmers produced fruit and vegetables in the Monzón area, in the northeast of Spain, to supply the population of nearby places. The famous local Caparrona beans reached a production higher than 200,000 kg and was commercialized in the Spanish national market (Raluy, 1982). The industrial development meant that most farmers no longer cultivated beans, and only a few local growers continued producing Caparrona beans mainly for family use. However, the high demand for local products, grown with environmentally friendly farming techniques, has reawakened interest in this local bean.

Despite its significance in the past, this landrace is neither cited in the Spanish legume catalog (Carravedo and Mallor, 2008), nor is it represented in the Spanish National Inventory, which includes the passport data of the accessions held *ex situ* in the collections of the public Spanish genebanks. From these aspects, it is evident that the Caparrona beans are currently being threatened by extinction. Thus, there is enough justification for the present study to proceed with the aim of assessing this landrace, which is at high risk of genetic erosion.

To recover the Caparrona bean crop, a study was conducted with the following tasks: (a) to collect samples and obtain traditional knowledge from local orchards; (b) to evaluate the phytosanitary state; (c) to determine differences in morphology and agronomic characteristics and nutritional values; (d) to select the sample with more favorable characteristics for commercial production purposes; (e) to provide good-quality seeds to local farmers for *in situ* production; and (f) to maintain seeds in the vegetable Spanish genebank (BGHZ-CITA) for long-term *ex situ* conservation. Consequently, the aim of this paper is to study the possibility of recovering the "Caparrona" common bean landrace for commercial purposes.

MATERIALS AND METHODS

Sample Collection and Traditional Knowledge

Bean samples were collected from local growers in Monzón (Huesca, Spain). The orchards were visited twice. During the first visit, the plants were in the flowering stage of development and leaf samples were collected to test for seed-borne viral diseases. After assessing the presence or absence of viruses, the seed samples were collected only from the growers of healthy plants, during the second visit at harvest time. These samples were then used to carry out the assays. Additionally, traditional knowledge regarding Caparrona beans was obtained from the growers using a questionnaire in which they were asked for agronomical practices and uses.

Seed-Borne Virus Monitoring

Leaf samples were monitored for the Bean Common Mosaic Virus (BCMV) and the Bean Common Necrotic Mosaic Virus (BCNMV), because they are the most prevalent seed-transmitted diseases for bean seed production (De Ron, 2015).

To identify the virus, leaf samples were tested serologically by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA), using specific BCMV and BCNMV polyclonal antibodies from Agdia®.

Samples were obtained from mother plants in order to select healthy seeds for the assay, and during the growing season to select healthy seeds for growers. After preliminary analyses in mother plants, accessions showing virus infections were excluded. Finally, four from the six accessions were chosen and used in the study. During the growing season, four DAS-ELISA tests were performed, specifically for samples that were obtained on July 8, July 24, August 26, and October 2, corresponding to 13 days after sowing that corresponded to DAS (first trifoliate fully expanded stage of development), 29 DAS (anthesis), 62 DAS (flowering), and 99 DAS (grain filling).

Experimental Design and Cultivation Practices

Four bean samples, named CAP01, CAP02, CAP03, and CAP04, were evaluated in a randomized complete block design with three replications at two locations: (1) Monzón (41° 54′ N, 0° 11′ E, 279 m.a.s.l.) with an average temperature from seedling planting to harvest of 21.5°C and total rainfall of 137.5 mm and (2) Montañana (41° 43′ N, 0° 48′ W, 222 m.a.s.l.) with an average temperature of 21.4°C and rainfall of 44.1 mm during the growing season. Each of the three replications or plots consisted of 40 plants that were transplanted into two 20 plant-rows with a row-to-row distance of 1.1 m. apart and within a row distance of 0.15 m, equivalent to a crop density of 6.1 plants m^{-2}.

Before planting, the soil was prepared by a rotary cultivator with a roller. The crops were initiated on 9 July, with seedlings cultivated in a greenhouse from 25 June. Plants were drip-irrigated as needed. Harvest was performed by hand at the end of October: in Montañana, 126 days after sowing, and in Monzón, 119 days after sowing. For pest and disease control, plants were sprayed with Abamectin and Spiromesifen for *Tetranychus urticae* and *Trialeurodes vaporariorum* control and Clortalonil and Tiram for *Alternaria* and *Botrytis* control. For weed control, hand weeding was done.

The plants were supported using sticks, in accordance with traditional practice in the geographical area, because of its indeterminate growth habit.

Evaluation Data

Data related to phenology, yield, and seed characteristics, including quantitative and qualitative traits, were recorded following some of the International Board for Plant Genetic Resources (IBPGR) *Phaseolus vulgaris* descriptor list (International Board for Plant Genetic Resources [IBPGR], 1982). Qualitative traits were estimated for each plot, while quantitative traits were estimated in the detailed number of individuals (seedlings, pods, or grains), as follows:

- Growth habit was determined following the Singh (1982) key for growth habit identification.
- Leaflet length was measured on 10 seedlings, on terminal leaflet of third trifoliate leaf from pulvinus to leaf tip.
- Flowers: color of standard and color of wings were observed in freshly opened flowers.
- Immature pod: length, width, and thickness (in millimeters); weight (in grams); cross-section (very flat, pear shaped, round elliptic, and figure of eight); curvature (straight, slightly curved, curved, and recurving); suture string (stringless, few strings, moderately stringy, and very stringy); and color (dark purple, red, pink, yellow, pale yellow with colored mottling or stripes; persistent green) were determined on 30 immature pods.
- Mature pod: length, width, and thickness (in millimeters); weight (in grams); and number of seeds per pod were determined on 30 mature pods.
- Grain qualitative traits: seed coat patterns (constant mottled, striped, rhomboid spotted, speckled, circular mottling, marginal color pattern, broad striped, bicolor, spotted bicolor, pattern around hilum, and other); brilliance of seed (matt, medium, and shiny); seed shape (round, oval, cuboid, kidney shaped, and truncate fastigiate); and seed colors were observed for each plot.
- Grain quantitative traits: length, width, and thickness (in millimeters) were estimated to calculate length/width and width/thickness relationships, corresponding to more or less rounded shapes or more or less elongated shapes. These parameters were determined on 30 grains.
- Phenology traits on a plot average basis: beginning of flowering (days from sowing until 50% of plants in each plot had at least one open flower); physiological maturity (days from sowing until 90% of plants had dry pods ready for seed harvest); and immature pods at harvest time (number and weight of immature pods) were recorded.
- Agronomic traits: each plot was harvested at physiological maturity stage individually, and the number of total pods, seeds per pod, and the dry weight of seeds were measured to calculate the dry seeds per plant (g/plant); dry seeds per area (kg/ha); the number of pods per plant; and the number of seeds per pod. The 100-seed weight was also calculated as the average of five measurements.

For the nutritional value analysis, the following parameters were determined: the moisture content, in an oven set at 100°C to constant weight; the ash content, by calcination in a furnace at 520°C; the protein content, quantified by the Kjeldahl method and a conversion factor of 6.25; the lipid content, by Soxhlet extraction, using petroleum ether as the extractor; and the carbohydrate content by subtracting the sum of the lipid, protein, moisture, and ash contents from 100 (Association of Official Analytical Chemists, 2005). The calorie value was also calculated in kilojoules (kJ).

Statistical Analysis

Data was studied by means of ANOVA using the SPSS statistical package. The results were expressed as the means ± standard deviation (SD). The statistical significance of the data was analyzed using univariate analysis of variance ($P < 0.05$), and a *post hoc* Tukey-b test was performed to construct homogeneous groups (One-way ANOVA; SPSS for Windows, version 16.0).

RESULTS

Traditional Knowledge

The results of the six interviews showed that the local growers, who continue producing Caparrona beans in the area of Monzón are elderly people, aged from 67 to 78, with an average age of 72 years. The traditional sowing date varied from middle June to the beginning of July, mainly by direct seeding, but also by planting of seedlings. Harvesting takes place from the beginning of November, but local weather heavily influences it. The bean plants are traditionally supported using sticks obtained from the nearest riversides. Flood or blanket irrigation is currently utilized, although some growers expressed interest in changing to the drip system. One of the growers practice organic farming. The Caparrona beans normally are consumed as dry seeds, but another traditional way of consumption is as "granaderas" beans. In that case, the pods are harvested before they dry, the beans are obtained from the immature pods and, unlike dry beans, these "granadera" beans do not need to be soaked before cooking.

Seed-Borne Virus Monitoring

All the leaf samples obtained from plants in the greenhouse after transplanting and tested on July 8 (13 DAS) showed negative results for both analyzed viruses. The next sampling date was carried out on 29 DAS and the CAP02 plants resulted positive

for BCMV virus in both locations. The 62 DAS test carried out resulted positive for BCMV in CAP02 and CAP04 plants. Finally, the last test performed, 99 DAS, corresponding to grain-filling stage, also resulted positive for BCMV in CAP 02 and CAP04 plants. Neither of the tested samples was positive for BCMNV virus. Following these results, CAP02 and CAP04 were discarded.

Growth Habit and Leaflet Length

All the plants presented a type IV climbing growth habit (**Figure 1**). Although this type of plant usually presents higher seed production, the management is more complicated because of the need for tutoring the plants. Samples were grouped in two clusters according to leaflet length, one formed by CAP01, CAP03, and CAP04 (11.3 ± 1.4 to 11.7 ± 1.3 cm) and the other one formed by CAP02 (10.1 ± 1.9 cm). This size was considered large (>9 cm), regarding the intervals established by Gill et al. (2014).

Flowers

All flowers presented white with lilac edge standard and white wings. Only one plant from the CAP03 sample presented a plant with flowers completely lilac, including standard and wings. The seeds from this plant were also different (brown seeds); so, it was considered an outside type plant.

Pods

All immature pods presented pear-shaped cross-section, green color, and were slightly curved, except for the CAP02 sample that presented curved pods (**Table 1**). No significant differences for quantitative traits related to immature pods were found among the four Caparrona accessions. Regarding mature pods, CAP01 presented higher values, while CAP02 was the lowest, mainly regarding the pod length.

Phenological Traits

The number of days from sowing to 50% flowering, plants varied between 57 days (CAP01 and CAP04), 62 days (CAP03), and 68 days (CAP04). The harvest was done at the same time in each location. All the samples showed less than 1.5% of immature pods when plants were harvested, except for CAP02 that presented a mean of 5.7% of immature pods at the time of harvesting.

Grains

All Caparrona samples produced white beans with a brown pattern around the hilum, medium brilliance, and oval shape

FIGURE 1 | Field trial of Caparrona de Monzón bean.

FIGURE 2 | Dry pods and grains of Caparrona de Monzón bean.

TABLE 1 | Mean values for selected pod-related parameters in four Caparrona de Monzón bean accessions (CAP01, CAP02, CAP03, and CAP04) grown in two locations (1: Montañana and 2: Monzón).

	CAP01	CAP02	CAP03	CAP04	*P*
Inmature pod (location 1)					
Length (mm)	12.2 ± 0.9	11.8 ± 1.2	12.3 ± 1.2	12.3 ± 1.2	0.387
Width (mm)	15.0 ± 1.2	14.7 ± 1.0	15.1 ± 1.3	15.3 ± 1.2	0.312
Thickness (mm)	7.2 ± 1.7	7.7 ± 1.5	7.3 ± 1.9	7.3 ± 1.9	0.607
Weight (g)	6.6 ± 2.9	8.0 ± 2.2	7.6 ± 2.9	7.6 ± 2.8	0.275
Mature pod (location 1)					
Length (mm)	9.5 ± 2.3 a	7.9 ± 1.5 b	9.0 ± 2.4 a	9.0 ± 2.2 a	0.000**
Width (mm)	12.2 ± 1.9 a	11.4 ± 1.5 b	11.2 ± 1.6 b	11.4 ± 1.6 b	0.001**
Thickness (mm)	9.5 ± 1.7	9.4 ± 1.5	9.3 ± 2.1	9.4 ± 1.9	0.924
Mature pod (location 2)					
Length (mm)	11.1 ± 1.6 a	9.6 ± 1.7 c	11.2 ± 1.7 a	10.4 ± 1.6 b	0.000**
Width (mm)	10.8 ± 1.3	10.9 ± 1.0	11.0 ± 1.1	10.9 ± 1.2	0.787
Thickness (mm)	10.8 ± 1.5 a	10.0 ± 1.4 b	10.8 ± 1.6 a	10.4 ± 1.4 ab	0.002**

*Values are means ± SD; n = 30. Means within rows followed by the same letter are not statistically different at 0.05 significance level in Tukey-b post hoc test. Significant P-values: *P < 0.05, **P < 0.01.*

TABLE 2 | Mean values for selected grain parameters in four Caparrona de Monzón bean accessions (CAP01, CAP02, CAP03, and CAP04) grown in two locations (1: Montañana and 2: Monzón).

	CAP01	CAP02	CAP03	CAP04	*P*
Grain (original sample)					
Length/width	1.30 ± 0.01 b	1.28 ± 0.02 b	1.38 ± 0.02 a	1.36 ± 0.02 a	0.000**
Width/thickness	1.27 ± 0.07 a	1.12 ± 0.06 c	1.28 ± 0.12 a	1.18 ± 0.10 b	0.000**
Grain (location 1)					
Length/width	1.35 ± 0.09 bc	1.32 ± 0.08 c	1.43 ± 0.10 a	1.37 ± 0.09 b	0.000**
Width/thickness	1.40 ± 0.10 a	1.16 ± 0.09 c	1.32 ± 0.12 b	1.30 ± 0.14 b	0.000**
Grain (location 2)					
Length/width	1.29 ± 0.08 b	1.28 ± 0.07 b	1.34 ± 0.10 a	1.32 ± 0.07 a	0.000**
Width/thickness	1.27 ± 0.08 a	1.12 ± 0.06 c	1.23 ± 0.09 b	1.22 ± 0.08 b	0.000**

*Values are means ± SD; n = 30. Means within rows followed by the same letter are not statistically different at 0.05 significance level in Tukey-b post hoc test. Significant P-values: *P < 0.05, **P < 0.01.*

TABLE 3 | Mean values for agronomic traits in four Caparrona de Monzón bean accessions (CAP01, CAP02, CAP03, and CAP04) grown in two locations (1: Montañana and 2: Monzón).

	CAP01	CAP02	CAP03	CAP04	*P*
Location 1					
Seed yield (kg ha^{-1})	5819.2 ± 163.2 a	4429.3 ± 548.1 b	5762.1 ± 165.7 a	5422.2 ± 29.8 a	0.002**
Seed yield (g plant^{-1})	96.9 ± 0.3 a	76.7 ± 9.0 b	96.7 ± 2.7 a	94.2 ± 0.49 a	0.000**
Pods per plant	32.9 ± 1.0 a	27.3 ± 3.2 b	31.7 ± 1.6 a	34.0 ± 1.3 a	0.004**
Seeds per pod	4.1 ± 0.3 ab	4.6 ± 0.1 a	4.4 ± 0.1 ab	4.0 ± 0.2 b	0.031*
100-Seed weight	71.4 ± 1.0 a	61.0 ± 3.2 b	68.8 ± 1.8 a	69.0 ± 1.3 a	0.001**
Location 2					
Seed yield (kg ha^{-1})	3508.4 ± 1465.8	3167.9 ± 719.7	4974 ± 1015.3	3698.1 ± 357.7	0.238
Seed yield (g plant^{-1})	33.4 ± 9.9	28.1 ± 4.9	39.0 ± 6.8	30.5 ± 2.4	0.683
Pods per plant	16.9 ± 3.7	17.1 ± 2.1	18.9 ± 5.6	17.0 ± 1.1	0.935
Seeds per pod	3.0 ± 0.5	2.9 ± 0.2	3.5 ± 0.5	2.9 ± 0.2	0.385
100-Seed weight	66.1 ± 3.8 a	56.9 ± 1.6 b	59.7 ± 2.2 ab	61.9 ± 1.9 ab	0.019*

*Values are means ± SD; n = 3. Means within rows followed by the same letter are not statistically different at 0.05 significance level in Tukey-b post hoc test. Significant P-values: *P < 0.05, **P < 0.01.*

TABLE 4 | Nutritional compositions of four Caparrona de Monzón bean accessions (CAP01, CAP02, CAP03, and CAP04) grown in Monzón (Huesca).

Sample	Moisture (g.100 g^{-1})	Ash (g.100 g^{-1})	Lipid (g.100 g^{-1})	Protein (g.100 g^{-1})	Carbohydrate (g.100 g^{-1})	Caloric value (kJ)
CAP01	7.89 ± 0.08 a	3.97 ± 0.04	1.53 ± 0.18	22.09 ± 0.23 b	64.53 ± 0.54	1529.1 ± 1.6
CAP02	7.83 ± 0.09 a	3.81 ± 0.02	1.66 ± 0.00	22.47 ± 0.03 ab	64.25 ± 0.04	1535.6 ± 1.2
CAP03	8.02 ± 0.07 a	3.93 ± 0.05	1.50 ± 0.01	22.65 ± 0.18 ab	63.91 ± 0.21	1526.8 ± 0.2
CAP04	7.27 ± 0.03 b	4.09 ± 0.11	1.45 ± 0.15	22.70 ± 0.01 a	64.50 ± 0.01	1535.7 ± 5.3
P	0.002**	0.053	0.414	0.046*	0.263	0.077

*Values are means ± SD; n = 3. Means within columns followed by the same letter are not statistically different at 0.05 significance level in Tukey-b post hoc test. Significant P-values: *P < 0.05, **P < 0.01.*

(**Figure 2** and **Table 2**). Data from original grains, collected from local growers and used for sowing, and grain, which were harvested from both locations, were compared. The length/width ratio was as expected for an oval seed shape in all accessions, with values higher than the unit, ranging from 1.28 (CAP02) to 1.43 (CAP03). On the contrary, the width/thickness ratio also corresponded to an oval cross-section, ranging from 1.12 (CAP02) to 1.40 (CAP01). The lower values of width/thickness grain ratio obtained for CAP02 indicated a shape more rounded, and slightly different to the rest.

Agronomic Traits

Significant differences were found between the two locations regarding agronomic traits (**Table 3**). The statistical analysis of the yield, estimated by seed yield in kg per ha, showed that this parameter depended on the seed sample (P = 0.008) and the location (P = 0.000). The interaction between both factors (sample x location) was not significant (P = 0.284). In that way, the Montañana plot was more productive than the one in Monzón, due to a better phytosanitary state of the plants. The obtained data showed higher values than the Spanish national mean of 1,899 kg ha^{-1} and similar to the mean values cited

in the region of 2,250 kg ha^{-1} (MAPAMA, 2017). According to the classification established by Asensio (2006), this local variety corresponded to a dry bean of high yield and a long life-cycle.

Nutritional Composition

Of all the studied samples, significant differences ($P < 0.05$) were observed only in moisture and protein contents, with sample CAP04 showing a lower moisture content and a higher protein content, although the differences were not very important (**Table 4**). Among the most remarkable compounds, common beans are noted for their protein content, being an excellent source of plant-based protein. The total protein content depends on the variety, and ranges from 16 to 33% (Oliveira et al., 2017). The results obtained for Caparrona beans are included in these limits and ranged from 22.1 to 22.7 g.100 g^{-1}. Similar values are reported by Rezende et al. (2017) in Brazilian beans (19 to 23 g.100 g^{-1}) or by Santalla et al. (1995) in a wide range of samples from Northwestern Spain (22 to 27 g.100 g^{-1}).

DISCUSSION AND CONCLUSION

The closely related potyviruses, BCMV and BCMNV, are major constraints to common bean (*Phaseolus vulgaris*) production (Worrall et al., 2015). The serological tests showed BCMV virus infection in CAP02 accession in the second sampling date and on CAP04 on the third sampling date. Symptomatic plants were found in the three replications in both locations. Symptoms consisted of mosaic and leaf deformations. Both accessions resulted in being less productive. Accessions CAP01 and CAP03 were virus free in all the tests done. Since the virus is transmitted in a non-persistent manner by aphids and none of the CAP01 and CAP03 plants were infected, this germplasm should be a useful source of genetic diversity for BCMV resistance, although further studies are necessary to confirm the genetic resistance.

Between the two healthy seed samples (CAP01 and CAP03), no significant statistical differences were found regarding morphological, nutritional, phenological and agronomic results. Both samples have been selected for commercial purposes, mainly due to high production and protein content.

Caparrona landrace has been classified as a high yield crop (Asensio, 2006). This fact increases the profit margin per unit area and consequently producers should be encouraged to grow Caparrona beans. Additionally, dry beans are extremely important for human nutrition. Lioi and Piergiovanni (2015) reported that seeds contain from 18 to 28% of proteins, being rich in lysine, which complements the nutritional profile of cereals and tubers. Caparrona beans contain from 22 to 23% of proteins, so it should be considered of nutritional interest for human consumption. The worldwide common bean production has significantly increased in the last three decades, except in Europe, where it has dropped (Lioi and Piergiovanni, 2015). Nevertheless, the recent inclusion of the Mediterranean diet in the UNESCO list of the "Intangible Cultural Heritage of Humanity," which emphasizes on pulse consumption and the recent proposal of common beans as a nutraceutical food should increase the human common bean consumption.

The obtained seeds from the assays were transferred to a recently created producers' association called "*Asociación de productores y dinamizadores de la Judía Caparrona de Monzón*," which registered a private label to commercialize the Caparrona beans. Seeds are also available from the BGHZ-CITA public genebank (FAO code ESP027). The accession is identified by the Genebank code BGHZ5788 and the National Inventory code NC105048.

This study provides a comprehensive model for *ex situ* and *in situ* landrace conservation from collection of local genetic resources to the recovery of Caparrona bean cultivation for commercial production. In that way, this study has allowed to: (1) describe the local bean Caparrona de Monzón using morphological, nutritional, phonological, and yield traits; (2) produce seeds with adequate quantity and quality (virus-free with high-germination rates) for *in situ* conservation by the local growers; and (3) guarantee the *ex situ* conservation in the Spanish public vegetable germplasm bank.

In summary, the local "Caparrona de Monzón" bean has been returned to the fields and now is produced and commercialized, mainly in the local area, as a gourmet product.

AUTHOR CONTRIBUTIONS

CM and JA conceived, designed, and wrote the manuscript. All authors contributed to analysis and analyzed the data.

FUNDING

This study was supported by CEDER Zona Oriental de Huesca, project 349-C 'Estudio y Caracterización de la Judía Caparrona de Monzón.'

ACKNOWLEDGMENTS

The authors want to express their gratitude to the local growers and donors of the seeds, especially to Jesús Raluy; the "Asociación de Productores y Dinamizadores de la Judía Caparrona de Monzón," presided by Javier de Pablo; and Enrique Playán for providing historical knowledge and data.

REFERENCES

Asensio, C. (2006). *Catálogo de Variedades de Judías-Grano del ITACyL*. Valladolid: Instituto Tecnológico Agrario y Junta de Castilla León, 36.

Association of Official Analytical Chemists (2005). *Official Methods of Analysis of the Association of Analytical Chemists International*, 18th Edn. Gathersburg, MD: U.S.A Official methods.

Barbieri, R. L., Costa, J. C., Alercia, A., and Padulosi, S. (2014). Agricultural biodiversity in Southern Brazil: integrating efforts for conservation and use

of neglected and underutilized species. *Sustainability* 6, 741–757. doi: 10.3390/su6020741

Cardador-Martínez, A., Loarca-Piña, G., and Oomah, B. D. (2002). Antioxidant activity in common beans (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* 50, 6975–6980. doi: 10.1021/jf020296n

Carravedo, M., and Mallor, C. (2008). *Variedades Autóctonas de Legumbres Españolas*. Zaragoza: Gobierno de Aragón.

De Ron, A. (ed.) (2015). *Grain Legumes*. New York, NY: Springer-Verlag, doi: 10.1007/978-1-4939-2797-5

Eurostat Statistic Explained (2017). *Agricultural Production-Crops*. Available at: http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production_-_crops (accessed April 12, 2018)

Gill, H. R., Rosales, R., Hernández Delgado, S., and Mayek, N. (2014). Morphological and molecular characterization of common bean landraces cultivated in the semi-arid Mexican high plateau. *Boletín Soc. Argentina Bot.* 49, 525–540.

International Board for Plant Genetic Resources [IBPGR] (1982). *Descriptors for Phaseolus vulgaris. International Board for Plant Genetic Resources – IBPGR*. Rome: IBPGR. doi: 10.1016/j.foodqual.2006.05.009

Kreutzmann, S., Thybo, A. K., and Bredie, W. L. P. (2007). Training of a sensory panel and profiling of winter hardy and coloured carrot genotypes. *Food Qual. Pref.* 18, 482–489. doi: 10.1002/jsfa.8558

Lioi, L., and Riergiovanni, A. R. (2015). Common bean cultivation in the Mediterranean Basin. *Legume Perspect.* 10, 22–24. doi: 10.1002/jsfa.8558

MAPAMA (2017). *Anuario de Estadística Agraria*. Available at: http://www.mapama.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/ doi: 10.1155/2017/5957178

Oliveira, A. P., Andrade, G., Mateó, B. S. O., and Naozuka, J. (2017). Protein and metalloprotein distribution in different varieties of beans (*Phaseolus vulgaris* L.): effects of cooking. *Int. J. Food Sci.* 8:5957178. doi: 10.1155/2017/5957178

Pearson, D., Henryks, J., Trott, A., Jones, P., Parker, G., Dumaresq, D., et al. (2011). Local food: understanding consumer motivations in innovative retail formats. *Br. Food J.* 113, 886–899.

Raluy, R. (1982). *Monzón mi Ciudad 1920-1950*. Zaragoza: Tipo Línea, S.A.

Reynoso-Camacho, R., Ramos-Gomez, M., and Loarca-Pina, G. (2006). "Bioactive components in common beans (*Phaseolus vulgaris*)," in *Advances in Agricultural and Food Biotechnology*, eds R. G. Guevara-González and I. Torres-Pacheco (Trivandrum: Research Signpost), 217–236. doi: 10.1590/1678-457x.05917

Rezende, A. A., Pacheco, M. T. B., da Silva, V. S. N., and de Castro Ferreira, T. A. P. (2017). Nutritional and protein quality of dry Brazilian beans (*Phaseolus vulgaris* L.). *Food Sci. Technol.* 38, 421–427. doi: 10.1590/1678-457X.05917

Richards, T., Hamilton, S. F., Gómez, M., and Rabinovich, E. (2017). Retail Intermediation and Local Foods. *Am. J. Agric. Econ.* 99, 637–659.

Santalla, M., Cardelle, M., Dans, C., and Barreiro, F. (1995). Valor nutritivo de la judía grano y su papel en alimentación humana. *Agricultura* 755, 464–469.

Santalla, M., Rodiño, A. P., and De Ron, A. M. (2002). Allozyme evidence supporting southwestern Europe as a secondary center of genetic diversity for common bean. *Theor. Appl. Genet.* 104, 934–944. doi: 10.1007/s00122-001-0844-6

Singh, S. P. (1982). A key for identification of different growth habits of *Phaseolus vulgaris* L. *Annu. Rep. Bean Improv. Coop.* 25, 92–94. doi: 10.1016/bs.aivir.2015.04.002

Worrall, E. A., Wamonje, F. O., Mukeshimana, G., Harvey, J. J., Carr, J. P., and Mitter, N. (2015). Bean common mosaic virus and bean common mosaic necrosis virus: relationships, biology, and prospects for control. *Adv. Virus Res.* 93, 1–46. doi: 10.1016/bs.aivir.2015.04.002

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 November 2018
doi: 10.3389/fpls.2018.01642

The Spanish Core Collection of Common Beans (*Phaseolus vulgaris* L.): An Important Source of Variability for Breeding Chemical Composition

Ana Rivera[1], Marçal Plans[2], Josep Sabaté[1,3], Francesc Casañas[1], Joan Casals[1,3], Aurora Rull[1,3] and Joan Simó[1,3]*

[1] Miquel Agustí Foundation, Barcelona, Spain, [2] Nestle Purina, St. Louis, MI, United States, [3] Department of Agri-Food Engineering and Biotechnology, BarcelonaTech, Universitat Politecnica de Catalunya, Barcelona, Spain

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Paul John Hunter, University of Warwick, United Kingdom
Caspar Christian Cedric Chater, University of Sheffield, United Kingdom

***Correspondence:**
Ana Rivera
ana.rivera@upc.edu

Specialty section:
This article was submitted to Agroecology and Ecosystem Services, a section of the journal Frontiers in Plant Science

Received: *07 June 2018*
Accepted: *23 October 2018*
Published: *13 November 2018*

Citation:
Rivera A, Plans M, Sabaté J, Casañas F, Casals J, Rull A and Simó J (2018) The Spanish Core Collection of Common Beans (Phaseolus vulgaris L.): An Important Source of Variability for Breeding Chemical Composition. Front. Plant Sci. 9:1642. doi: 10.3389/fpls.2018.01642

The Iberian Peninsula is considered as a secondary center of diversity for the common bean, and the Spanish National Plant Genetic Resources Centre's germplasm bank holds more than 3,000 Spanish accessions of *Phaseolus vulgaris* L. from which a core collection of 202 landraces has been selected. In order to encourage the use of this abundant resource, this study aimed to characterize genetic diversity, by measuring chemical composition in these core collections (in both the seed coat and cotyledon) using previously developed near infrared spectroscopy models. Crucially, these landraces in question all originated under similar agroclimatic conditions, allowing these field trials to be conducted in a single location without significantly altering the agronomic behavior of individual accessions. Using previously reported data, we also explored the correlations between chemical composition and culinary/sensory traits, as well as possible associations between chemical composition and seed coat color or gene pool (Middle American or Andean). The general Mahalanobis distance was >3 in only 11 of 1,950 estimations, confirming the robustness of the regression models previously developed. Variability was greater in seed coat than in cotyledon compounds and ranges for all compounds were wide: ash 34–94 g/kg, Ca 5–31 g/kg, dietary fiber 554–911 g/kg, Mg 2–4.4 g/kg, uronic acid 95–155 g/kg, protein 192–304 g/kg, starch 339–446 g/kg, amylose 208–291 g/kg, amylopectin 333–482 g/kg, and apparent amylose 241–332 g/kg. Accessions with white seed coats tended to be richer in ash, dietary fiber, uronic acid, and Ca, and accessions of the Middle American gene pool had on average 65% more Ca than the Andean gene pool. Strong genetic correlations were not identified between chemical and culinary/sensory traits. This is particularly positive with regards to plant breeding, as it means that synchronic improvement of nutritional composition and sensory traits is possible. The genetic diversity of chemical composition described in the Spanish core collection of beans therefore represents a promising opportunity to develop cultivars with superior nutritional profiles.

Keywords: *Phaseolus vulgaris*, common bean, diversity, genebanks, gene pool, nutrient composition, protein concentration, seed color

INTRODUCTION

Dry beans (*Phaseolus vulgaris* L.) are an extremely important aliment, not only representing the main source of dietary protein for humans in several world regions but also contributing greatly to diet with starch, fiber, vitamins, and minerals (Hayat et al., 2014). Annual global production is presently approximately 26.5 million tons, most of which is used for human consumption (FAOSTAT, 2018). Although in the most developed countries, the importance of dried bean consumption has diminished with the increase of meat consumption, producing animal protein is far more expensive, and is generally considered unsustainable (Pimentel and Pimentel, 2003; Gonzalez et al., 2011). Red meat in particular also has negative effects on health, for example being associated with the development of cardiovascular disease and cancer (Micha et al., 2010; Pan et al., 2013; Gonzales et al., 2014; Ekmekcioglu et al., 2018). International organizations and specialists therefore recommend increasing the consumption of beans and other legumes to fulfill our nutritional needs and decrease inputs of food production (Leterme, 2002; WHO, 2015; FAO, 2016; De Ron et al., 2017; McDermott and Wyatt, 2017). To promote this change in diet, apart from optimizing crop yields, we also need to identify palatable varieties of beans and legumes that are both tied to our gastronomic cultures and provide the maximum amount of nutrients (especially protein). Breeding programs for beans have focused mainly on maximizing yield (Kelly et al., 1998; Singh, 1999) and improving resistance to both biotic and abiotic stresses (Ishitani et al., 2004; Miklas et al., 2005; Singh and Schwartz, 2009; Araújo et al., 2015). More recently, however, nutritional and sensory traits have also been added to the ideotypes of these crops (Vaz Patto et al., 2015).

Natural genetic variation for chemical composition in the cultivated genepool and wild crop relatives of *Phaseolus vulgaris* is particularly important as it offers us the opportunity to develop cultivars with superior nutritional profiles. For example, reported percentages of protein content range from 18 to 31%, from 50 to 76% for carbohydrates, and from 0.05 to 0.31% for Calcium (Ca) (Sathe et al., 1984a; Islam et al., 2002; Pinheiro et al., 2010; Hayat et al., 2014). This genetic diversity can fortunately be found conserved in agrosystems located in the centers of diversity for the species (*in situ* conservation) and in genebanks (*ex situ* conservation). Of more than 7.4 million accessions stored in approximately 1,750 genebanks (FAO, 2010), 260,000 belong to *Phaseolus vulgaris* L. Although significant efforts have been devoted to characterizing such germplasm collections for simple traits (mostly related to botanical aspects) (Wang et al., 2017) and for the most important agronomic traits (yield and resistance to pests and diseases) (Tanksley and McCouch, 1997), less is known about other important characteristics such as those related to nutritional, culinary, or sensory attributes (Smýkal et al., 2015). This is mainly due to the fact that these traits are quantitative and multigenic, with low heritability and strong genotype by environment interactions (GxE), thus making it difficult to assign a genotypic value for each accession. Moreover, the phenotyping of these traits is expensive in terms of both direct monetary input and human labor. However, the lack of phenotyping data available, regarding the accessions conserved *ex situ*, hinders utilization of this genetic diversity both in breeding programs and directly by farmers (Ramanatha Rao and Hodgkin, 2002; Hodgkin et al., 2003). Novel "omics" platforms enabling the massive analysis of the genome, transcriptome, proteome, and metabolome (Tanksley and McCouch, 1997; Prada, 2009; Langridge and Fleury, 2011), and information science (Michael et al., 2018), represent promising tools to find desirable agronomic alleles in seed banks and unlock the stored genetic diversity. The development of core collections that are representative of the genetic spectrum in the whole collection (van Hintum et al., 2000) has been proposed as an alternative to enable cost-effective characterization of the plant genetic resources held in genebanks. Initially, complex traits are phenotyped in the core collection, and if desirable qualities are found, similar accessions can then be identified in the whole collection (Prada, 2009). An example of this strategy is the Spanish core collection of beans of the Spanish National Plant Genetic Resources Center (CRF), comprising 202 accessions representative of the variability present in the total set of accessions collected in Spain. This collection was compiled based on location data (province, township, and altitude) and seed phenotype (color, shape, and size) (De la Rosa et al., 2000) and is an important resource because the Iberian Peninsula is considered a secondary center of diversity for *Phaseolus vulgaris* L. (Santalla et al., 2002). To date, several traits have been measured in this collection: flower, pod, and seed traits; growth habit; type of phaseolin and molecular markers (Pérez-Vega et al., 2009); resistance to pests and diseases [including halo blight (*Pseudomonas syringae*), common bacterial blight (*Xanthomonas campestris*) (Asensio et al., 2010), anthracnose (*Colletotrichum lindemuthianum*) (Pérez-Vega et al., 2006), and white mold (*Sclerotinia sclerotiorum*) (Pascual et al., 2010)]; and sensory and culinary traits (Rivera et al., 2016). However, nothing was known about the variability in chemical composition and nutritional potential of this collection. The main factors generally limiting substantial chemical composition studies and comparison of materials conserved in genebanks are the difficulties involved in analyzing so many samples. Nowadays, however, instrumental methods such as near infrared spectroscopy (NIRS) or nuclear magnetic resonance spectroscopy (Hacisalihoglu et al., 2010; Parlak and Güzeler, 2016) make it possible to analyze large collections of samples. With this objective, our team has developed NIRS predictive models for chemical composition and sensory traits (Plans et al., 2012, 2013, 2014), which show a robust capacity for accurate prediction.

The present study extends our previous work about the culinary and sensory traits of the accessions in the CRF's core collection of common beans (Rivera et al., 2016). Here, we examine the collection to (i) describe the variability of chemical composition in the seed coat and cotyledon, (ii) compare and correlate this variability and its magnitude with the variability in sensory and culinary traits described in the previous study, and (iii) analyze the possible associations between chemical composition and seed coat color and the Middle American or Andean gene pool.

FIGURE 1 | Photograph showing the variety of shapes and colors represented in the Spanish core collection of beans.

MATERIALS AND METHODS

Plant Material and Field Trials

The 202 accessions from the Spanish core collection of common beans form a rich mosaic of colors and shapes (**Figure 1**), encompass 51 market classes (Santalla et al., 2001), and represent all areas of Spain where beans are cultivated. All of the seeds were sown in Sabadell (Northeast Spain: 41° 32′ 50.7″ N, 2° 4′ 14.7″ E), a location with loam soil with abundant Ca (6.35 mg/kg), low phosphorus (6 mg/kg), and a mild Mediterranean climate, which allows both short and long-cycle accessions to develop to maturity. We used a randomized two block design with 21 plants per block and accession (total 42 plants per accession). The experiment was conducted at a low density (29,167 plants/ha) to facilitate the individualized recording of data. A vertical plastic net was used to trellis the accessions with indeterminate growth. All plants were cultivated with the traditional management practices of the area, including drip irrigation and fert-irrigation application (NPK 110 kg/ha). Due to the lack of significance of the block factor for agronomic traits (data not shown), seeds from the two blocks were pooled in order to ensure sufficient sample was available for the analyses. Seeds from a total of 195 accessions were harvested and processed for analyses (seven accessions did not produce enough seeds for analyses).

Sample Preparation

The seed coat accounts for less than 10% of the total dry matter of bean seeds and has a chemical composition distinctly different to that of the cotyledon (Singh et al., 1968; Moraghan et al., 2006). To ensure accurate information about the two fractions, seed coat, and cotyledons were analyzed separately. Seeds from each harvested accession (approximately 50 g) were soaked in 150 ml distilled water for 24 h, drained, dried with filter paper, and weighed. The seed coat was then manually separated from the cotyledon, and the two fractions were dried (60°C for 48 h). Finally, dried samples were ground to 0.4 mm in a laboratory mill (Perten 3100, Perten Instruments Inc., Springfeld, IL, United States). Ground samples were stored in polyethylene bags at 4°C until spectroscopic analysis was conducted.

NIRS Recording to Estimate Chemical Composition

Near infrared spectroscopy is a well-established technique for determining the components of foods (Nicolaï et al., 2007). Models developed by means of multivariate analysis correlating physicochemical properties, and the spectra obtained with NIRS technology enable prediction of the value of a sample. In our study, we used NIRS predictive models previously developed by Plans et al. (2012), (2013), to estimate the following chemical components: ashes, Ca, magnesium (Mg), dietary fiber, and uronic acids in the milled seed coat; and protein, starch, amylose, amylopectin, and apparent amylose in the milled cotyledon. These traits were selected because of their relationship with sensory attributes driving consumer preferences, as reported in previous studies (Casañas et al., 2002, 2006; Mkanda et al., 2007). All results were expressed as g/kg dry matter. The estimations were calculated using regression models from NIRS measurements. Infrared spectra from the ground samples were recorded by a spectrophotometer (model 5000, Foss NIRSystems, Silver Spring, MD, United States) at every 2 nm between 1,100 and 2,500 nm, with a mean of 32 scans performed. Each sample was analyzed per triplicate, and the mean spectrum reading was used for calculations. The absorbance of each wavelength was transformed into Log (1/*R*), where *R* represents reflectance, due to this variable correlating better with chemical components than raw reflectance. To record spectra and import data, VISION software was used (version 2.51, Foss NIRSystems, Silver Spring, MD, United States).

Reference Analysis

Twenty out of the accessions under study were randomly selected in order to perform chemical analysis. For each accession, three biological replicates were characterized for the chemical components. Ash, Ca, dietary fiber, Mg, and uronic acid were quantified by reference methods following the methodology described in Plans et al. (2012), while protein, starch, amylose, amylopectin, and apparent amylose were analyzed as described in Plans et al. (2013). These reference analyses were used to increase the domain of the chemical constituents of the NIRS model, thus improving its robustness as described by Barton et al. (2000).

Calculating Chemical Composition

Two different approaches can be used to correlate NIRS data from samples with the constituents of interest (in this case, chemical composition): global models based on partial least squares regression (PLSR) and principal components

regression (PCR) or local models based on the similarity of the unknown spectrum to the known spectra in a database. Classical regression techniques such as PLSR and PCR assume that the relationship between spectral and reference constituents is linear (Shenk and Westerhaus, 1991). The accuracy of NIRS in predicting chemical constituents is related to the structure and distribution of spectra in the calibration set, which must cover most of the possible variability in spectra encountered during routine analysis. For global PLSR models, increasing the domain of the calibration equation to include new diverse geographical and climatic areas, will require a larger number of representative samples in the calibration set. This, in turn, increases the complexity of the spectral reference model, making it necessary to compute more parameters to explain the variation in the constituents (Martens and Naes, 1989). The accuracy of prediction using global PLSR models usually decreases when the domain of the constituent increases.

Local calibration, also called memory-based regression, aims to overcome these difficulties. Since, in theory, an optimal prediction should be computed based on a specific calibration equation, we can use a small calibration dataset tailored to the unknown sample from a large database of spectra. This approach combines the advantage of global calibration (to cover a large constituent domain) and the accuracy obtainable with specific calibrations based on spectral similarities of a smaller set of samples. Various approaches can be used to calculate local models, with the most common algorithms being the classic similarities based on Mahalanobis distances (H) (Naes et al., 1990) and correlation coefficients (Shenk et al., 1997). Several authors found that local regression models significantly improved (20–30%) the standard error of prediction (SEP) compared to global calibration (Naes et al., 1990; Aastveit and Marum, 1993; Sinnaeve et al., 1994) in studies using sample selection and calibration methods based on PCR and PLSR.

We used global PLSR-derived correlations between NIR spectra and reference constituents (cotyledon: protein, starch, amylopectin, amylose, and apparent amylose; and seed coat: ash, calcium, dietary fiber, magnesium, and uronic acid). These models were previously developed using a wide range of genetic and environmental variability (with cultivation in many locations) (Plans et al., 2012, 2013). Furthermore, we increased the domain of the chemical constituents by using a set of 20 new accessions from the Spanish core collection of common beans. To avoid the problems inherent in using PLSR with increased domains, we used local regression (Ramirez-Lopez and Stevens, 2016) to correlate the spectra with the chemical referents. Local models were not developed using a single PLSR or PCR equation; rather a new calibration was created when an unknown sample was presented against the spectral database. The ability to predict unknown samples was based on global Mahalanobis distances (GH). This distance is normalized when measured in standard deviation units from the center of the selected set of samples. Based on a normal distribution, samples with a GH of greater than three would be classified as outliers and predictions in these samples would be considered questionable (Shenk et al., 1997).

Statistical Analysis

To estimate the global variability for each trait, and the relationships between traits, we calculated their mean, and variance. Previous results on culinary, sensory, and external appearance traits obtained from the same samples (Rivera et al., 2016) were used to calculate the correlation with the results obtained in this study. Sensory traits were evaluated using regression models from NIRS measurements in ground cooked samples. These models were previously developed by using evaluations from a trained panel (Plans et al., 2014). By contrast, culinary traits were quantified directly by using a standardized cooking process, as described in Romero del Castillo et al. (2012). Correlation analysis was performed using the Pearson coefficient with the Bonferroni correction. For further analysis of the variability, the accessions were classified according to their seed coat color and origin gene pool (Middle American or Andean). The data concerning seed coat color were reported by Rivera et al. (2016). Seeds were visually evaluated and divided into two categories: white and colored (including: yellow, cream, gray brownish to greenish, brown, vinous brown, black, ochre, purple, rosy, bicolor, and tricolor). The origin gene pool was previously described by PérezVega et al. (2009), following study of the phaseolin protein pattern and 11 molecular markers [two sequence characterized amplified regions (SCAR) and nine simple sequence repeats (SSR)] in the collection. For each type of classification, analysis of variance (ANOVA) was carried out based on the linear model $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where y_i was an individual level for a specific trait; μ was the grand mean; α_i was the effect of i group based on the type of seed coat color, or gene pool origin; and ε_{ij} was the random error for i groups with j replications of the model following a $N\sim(0, \sigma^2)$. All factors were considered to be fixed. Finally, for seed coat color and gene pool classification, we performed normalized principal component analyses (PCA) and calculated 95% confidence ellipses around each cluster of accessions with the same category. We used R software (R Core Team, 2017); agicolae, PCAmethods, and ellipse packages for all statistical analyses (Murdoch and Chow, 2007; Stacklies et al., 2007; Mendiburu, 2017).

RESULTS

Overall Variability in the Collection

The GH values obtained were extremely low and reflect the reliability of our estimations using the NIRs models. Only 11 from a total of 1950 estimations presented a GH value greater than 3. These values corresponded to amylopectin, and in each case were not considered in the subsequent computations. Furthermore, the mean GH values for individual traits all scored below GH < 1 (except amylopectin; GH < 1.3), thus highlighting the robustness of our data regarding the different chemical components in both the seed coat and cotyledon (Shenk et al., 1997).

The proportion of seed coat ranged from 4% to 10% (coefficient of variation, CV = 11.45%), and the proportion of cotyledon ranged from 90% to 96% (CV = 0.91%). For the chemical traits measured in the cotyledon, the CV ranged from

5.35% for starch to 10.97% for proteins. In general, CV from the seed coat components measured is higher than the CV from the cotyledon. In the seed coat, CV ranged from 8.31% for uronic acids to 39.07% for Ca (**Table 1**).

Mean phenotypic values were used to estimate genotypic correlations among chemical compounds (**Table 2**). In the cotyledon components, negative correlations were identified between protein and starch (−0.60, $p < 0.001$), amylose (−0.37, $p < 0.001$), and apparent amylose (−0.34, $p < 0.01$). Positive correlations were identified between many other traits, the most significant being between amylose and apparent amylose (0.69, $p < 0.001$), and between starch and amylose (0.5, $p < 0.001$). With regard to the seed coat composition, several significant correlations were found, including between Ca and ashes (0.91, $p < 0.001$), uronic acid and dietary fiber (0.42, $p < 0.001$), and uronic acid and ashes (0.35, $p < 0.01$). In relation to correlations between seed coat and cotyledon compounds, no correlations were identified. Considering the wide range of genetic diversity explored, we also performed a correlation analysis between the chemical results obtained in this work and both sensory and culinary traits reported previously in the same samples (Rivera et al., 2016). This analysis included 174 out of the original 195 accessions, because for some accessions there were not enough available seeds to perform the sensory and culinary analysis, for which a large sample is needed. Several significant correlations were identified (**Table 2**). With regard to sensory traits, the most significant correlations were found between mealiness and the seed coat measurements of ashes, Ca, dietary fiber, and uronic acid (−0.62, −0.57, −0.42, and −0.47, $p < 0.001$, respectively), and between seed coat brightness and ashes, Ca, dietary fiber, and uronic acid (0.52, 047, 0.43, and 0.46, $p < 0.001$, respectively). Fewer significant correlations were found with the culinary traits, for instance between rate of water absorption and ashes, Ca, dietary fiber, and uronic acid (0.39, 0.36, $p < 0.001$; 0.33 $p < 0.01$; and 0.3, $p < 0.05$, respectively), and between cooking time and protein (0.33, $p < 0.01$). Percentage of white surface color was positively correlated with all chemical components measured in the seed coat, while 100 seed weight correlated only with Ca (−0.37, $p < 0.001$) and ashes (−0.34, $p < 0.01$) (**Table 2**).

Effect of Seed Color on Chemical Variability

Seed color description, as reported in Rivera et al. (2016), revealed that 38 out of the 174 accessions studied had white seeds, and 136 accessions presented colored seeds. An ANOVA comparing the seed color classification (white or colored) was performed for all of the chemical traits measured (**Table 3**). Significant differences were found between white and colored seeds for all of the traits measured in the seed coat (ash, Ca, dietary fiber, Mg, and uronic acid), while solely for starch content regarding the traits measured in the cotyledon. White seeds tended to have higher concentrations for all of the traits measured in the seed coat and had lower starch content. The PCA created, considering all of the chemical traits evaluated in the seed coat and cotyledon with the different representation of the white and colored accessions, showed that the distribution of the accessions along the first (PC1) and second (PC2) principal components together explain 49% of the total variation (**Figure 2**). PCA showed that ash, Ca, uronic acids, and dietary fiber, which were correlated with PC2, had the higher contribution for clustering between groups, while the other chemical compounds had a low influence. Nevertheless, the confidence intervals for the two color groups overlapped, highlighting that within the colored group, there are genotypes with high values for the chemical traits analyzed.

Effect of Andean or Middle American Gene Pool on Chemical Variability

Previously, PérezVega et al. (2009) had used phaseolin and molecular markers to classify the Spanish core collection according to their origin gene pool. Forty three out of the 174 accessions studied were classified within the Middle American gene pool, and 131 within the Andean gene pool. Using this classification, we performed an ANOVA, which revealed significant differences among gene pools for the following components: in the cotyledon, protein, starch ($p < 0.001$), amylose, and apparent amylose ($p < 0.01$); in the seed coat, ashes, and Ca ($p < 0.001$) (**Table 4**). Accessions classified in the Middle American gene pool tended to have higher ash, Ca, and protein contents, with lower levels of starch, amylose, and apparent amylose. The greatest difference was found for the content of Ca in the seed coat, with Middle American samples yielding a content 65% higher than Andean materials (**Table 4**). The same PCA as **Figure 2** performed considering all of the chemical traits measured in the seed coat and cotyledon, but with the different representation of the accessions of Middle American and Andean gene pool showed that ash and Ca, which were correlated with PC2, had the higher contribution for clustering between groups (**Figure 3**). This multivariate analysis highlights that within the Andean gene pool there are genotypes with high values for uronic acids and dietary fiber contents (11 and 14 out of the 20 higher scores for uronic acids and dietary fiber, respectively, belong to accessions from Andean gene pool).

DISCUSSION

Use of NIR in this study enabled significant, in depth evaluation of the chemical composition of the Spanish core collection of beans. Data obtained here have added further knowledge to previously reported findings, helping make this one of the most well studied and characterized bean and legume collections. Detailed information, including pictures of seeds and flowers for each accession can be found on the website of the Spanish National Plant Genetic Resources Center: http://www.crf.inia.es/crfesp/paginaprincipaljudia.asp (verified 20 June 2018).

The chemical analysis performed on 20 samples enabled the inclusion of new reference values to the NIR models (Plans et al., 2012, 2013), thus increasing their robustness, as described by Barton et al. (2000), who showed the effect of adding new samples (∼10) to the memory based models reduced ¼ the SEP of the predictions (Barton et al., 2000). Accordingly, the prediction of all the components with the improved NIR models showed good precision, as for all the traits under study GH was below 3, which

TABLE 1 | Variability for the chemical compounds analyzed in the cotyledon and in the seed coat in the Spanish bean core collection of beans.

	Cotyledon components					Seed coat components				
	Protein	**Starch**	**Amylose**	**Amylopectin**	**Apparent amylose**	**Ashes**	**Ca**	**Dietary fiber**	**Mg**	**Uronic acids**
Mean	232.48	396.05	263.01	407.26	295.71	53.08	13.02	710.06	2.64	122.53
SD	25.49	21.19	22.60	35.42	18.30	11.32	5.09	72.36	0.42	10.18
SEM	1.83	1.52	1.62	2.54	1.31	0.81	0.36	5.18	0.03	0.73
Minimum	192.38	339.64	208.00	333.67	241.33	34.26	5.15	554.49	1.97	95.43
Maximum	304.24	446.53	291.00	482.00	332.81	94.27	30.74	911.22	4.47	154.77
CV	10.97	5.35	8.59	8.70	6.19	21.33	39.07	10.19	15.99	8.31

Each accession was analyzed per triplicate. Values are expressed in g/kg dry matter. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation (in %).

TABLE 2 | Genotypic correlations (Pearson coefficient with Bonferroni correction) between chemical composition traits measured in the cotyledon and seed coat, and sensory and culinary traits measured in a previous study (Rivera et al., 2016) using the same samples.

	Protein	**Starch**	**Amylose**	**Amylopectin**	**Apparent amylose**	**Ashes**	**Ca**	**Dietary fiber**	**Mg**	**Uronic acids**
Seed coat brightness	−0.08	0.04	0.11	0.12	0.15	0.52***	0.47***	0.43***	0.22	0.46***
Seed coat roughness	0.14	−0.3*	−0.11	−0.03	−0.05	0.37***	0.38***	0.22	0.2	0.25
Seed coat perception	−0.15	0.27	0.21	−0.02	0.32**	−0.29	−0.28	−0.19	0.01	−0.11
Mealiness	−0.06	0.2	0.09	−0.11	0.09	−0.62***	−0.57***	−0.42***	−0.15	−0.47***
Flavor	−0.03	0.26	0.21	−0.12	0.22	−0.35**	−0.3*	−0.18	−0.03	−0.16
Aroma	−0.07	0.37***	0.28	−0.12	0.23	−0.35**	−0.33**	−0.26	−0.01	−0.13
Rate of water absorption	0.04	−0.11	−0.07	0.06	−0.09	0.39***	0.36***	0.33**	0	0.3*
Water absorption during cooking (%)	0.21	−0.11	−0.02	−0.12	−0.03	0.25	0.21	0.16	0.11	0.17
Cooking time (min)	0.33**	−0.14	−0.09	−0.13	−0.04	0.06	0.08	0.02	0.1	0
Whole beans (%)	0.09	−0.11	−0.04	0.01	−0.24	−0.21	−0.2	0.06	−0.06	−0.11
White surface color (%)	0.05	−0.03	−0.02	0.07	0.03	0.57***	0.52***	0.47***	0.33***	0.53***
100 seed weight (g)	−0.06	0.13	0.09	−0.09	0.07	−0.34**	−0.37***	0.03	0	0.01
Protein		−0.6***	−0.37***	−0.22	−0.34**	0.05	0.08	−0.09	0.1	−0.15
Starch			0.5***	0.24	0.44***	−0.13	−0.1	0.03	−0.16	−0.04
Amylose				0.33*	0.69***	−0.08	−0.02	−0.06	−0.03	−0.13
Amylopectin					0.33**	0.09	0.07	−0.03	−0.02	−0.05
Apparent amylose							−0.01	0.06	−0.21	0.02
Ashes							0.91***	0.27	0.09	0.35**
Ca								0.2	0	0.21
Dietary fiber									0.06	0.42***
Mg										0.25

*Each accession was analyzed per triplicate. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.*

has been described as the threshold for considering a prediction robust (Shenk et al., 1997).

All the accessions showed a good agronomic behavior, completing the crop cycle, and enabling the harvesting of seeds. This was probably because all the accessions are landraces originating in similar agroclimatic conditions to those of the experimental field, thus yielding a similar phenotype to that of their area of origin. We identified great variability among the accessions of the core collection in some important chemical traits analyzed in the seed coats and cotyledons. The coefficients of variation were low in some cases [e.g., starch, amylose, amylopectin, apparent amylose, and uronic acid all had CV below 10% (**Table 1**)]. However, even in these cases, the extreme values were found to be far apart (with the maximum value at least 30% higher than the minimum for all of these traits). The proportion of seed coat ranged from 4% to 10%, CV (11.45%). These results are in accordance with those reported by Singh et al. (1968) and PérezVega et al. (2009), and slightly different from the variability described by Moraghan et al. (2006).

The few studies that have analyzed the chemical composition of the seed coat and cotyledon separately have reported values within the ranges found in this study (Singh et al., 1968; Moraghan et al., 2006). We were unable to find other studies that differentiated between components in the seed coat and cotyledon, but as the seed coat represents only a small proportion of the total weight of the beans, we can assume that the amounts found for the analytes in the cotyledon are not very different from what would be found in an analysis of

TABLE 3 | Analysis of variance to compare the levels of the different chemical components measured in the cotyledon and seed coat according to seed coat color classification proposed in Rivera et al. (2016).

	Cotyledon components					Seed coat components				
	Protein	Starch	Amylose	Amylopectin	Apparent amylose	Ashes	Ca	Dietary fiber	Mg	Uronic acids
Significance	ns	**	ns	ns	ns	***	***	***	**	***
White seeds	236.10	387.87	256.50	405.90	293.10	65.71	17.84	774.51	2.82	131.30
Colored seeds	229.80	399.46	264.60	406.48	296.63	50.61	11.91	696.47	2.59	120.34

****$p < 0.001$; **$p < 0.01$; ns, not significant. White seeds, 38 accessions; colored seeds, 136 accessions. Each accession was analyzed per triplicate. Values are expressed in g/kg.*

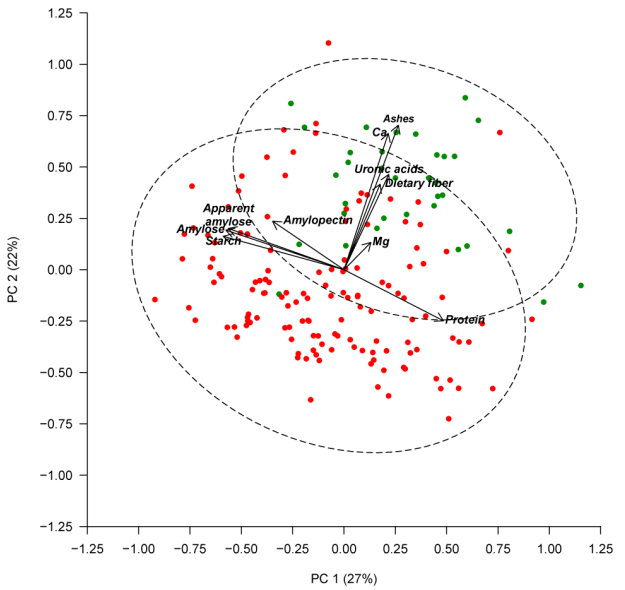

FIGURE 2 | Principal component analysis based on chemical composition of the seed coat and cotyledon. Accessions with white seeds are represented with green symbols and accessions with colored seeds are represented with red symbols. The dashed lines represent the 95% confidence ellipses.

the entire seed. The genetic differences identified for proteins and carbohydrates are within the range reported in studies considering the whole species (Sathe et al., 1984a,b; Hayat et al., 2014), and somewhat larger than those reported in specific collections (Paredes et al., 2009). Thus, it seems that the Spanish core collection represents a rich source of genetic variation for chemical composition in beans. Moreover, this diversity is present in genotypes that show good adaptation to the agroclimatic conditions of the Iberian Peninsula. Considering that this collection has been characterized by several traits, specific accessions can be selected for their agronomic, chemical, and sensory profiles, and used as elite genotypes to perform breeding programs devoted to obtain new varieties with good agronomic behavior, high contents of nutritional compounds, and sensory profiles close to consumer demands. This strategy can promote the consumption of this legume and achieve the objective of increasing the proportion of vegetable protein in the human diet.

With the aim of determining whether correlations exist between the assessed parameters which could potentially limit the progress in breeding programs for quality in common beans, we conducted a correlation analysis considering all of the traits studied in the cotyledon and seed coat. In this study, we identified significant positive correlations among the analyzed compounds in the seed coat, such as Ca and ash (0.92, $p < 0.001$). Moreover, results showed a significant correlation between protein and carbohydrates. These results are in accordance with Vargas-Torres et al. (2004) and Casañas et al. (2013), and imply that an increase in the concentration of protein leads to a decrease in starch content. No correlations have been found between the chemical composition of the seed coat and cotyledon, signaling that the chemical composition of both parts seems to be under independent genetic control. In accordance with this hypothesis, Casañas et al. (2013) identified independent quantitative trait loci (QTL) controlling chemical composition of the seed coat and cotyledon.

With regards to the relationship between chemical composition and sensory characteristics, it seems that ashes and Ca are the components with the strongest influence. Relative to the culinary traits, some authors have reported a tendency for genotypes with shorter cooking times to retain higher nutrient concentrations than those with longer cooking times (Wiesinger et al., 2016). In this work, the only relationship with cooking time was observed for protein. The lack of data for phytate content in the collection is an important shortcoming to complete this analysis, as this compound shows a strong relation with the culinary or sensory traits in beans (Casañas et al., 2002; Coelho et al., 2007). Our results also showed a negative correlation between Ca and weight of 100 seeds (−0.37, $p < 0.001$). In a study of eight accessions, Moraghan and Grafton (2001) reported a much higher negative correlation between these two characteristics. However, the diversity in Ca concentration observed in some accessions with a similar 100 seed weight highlights that there are other factors involved in determining this characteristic, such as the color of the seeds (**Table 2**) or the seed coat area/cotyledon weight ratios (Moraghan and Grafton, 2001).

Although there were many significant genotypic correlations between chemical components and culinary or sensory traits, most were not strong enough to make indirect selection efficient (i.e., the selection for a desired trait using another trait that is genetically linked). These findings contrast with those of studies

TABLE 4 | Analysis of variance comparing the content of the different analytes studied according to the gene pool classification reported by PérezVega et al. (2009).

	Cotyledon components					Seed coat components				
	Protein	Starch	Amylose	Amylopectin	Apparent amylose	Ashes	Ca	Dietary fiber	Mg	Uronic acids
Significance	***	***	**	ns	**	***	***	ns	ns	ns
Middle American	242.22	384.35	253.89	407.64	289.52	65.67	18.57	710.98	2.72	123.76
Andean	227.11	401.45	266.04	406.69	298.21	49.70	11.31	711.88	2.60	122.24

****$p < 0.001$; **$p < 0.01$; ns, not significant. Forty-three accessions classified within the Middle American gene pool, and 131 within the Andean gene pool. Each accession was analyzed per triplicate. Values are expressed in g/kg dry matter.*

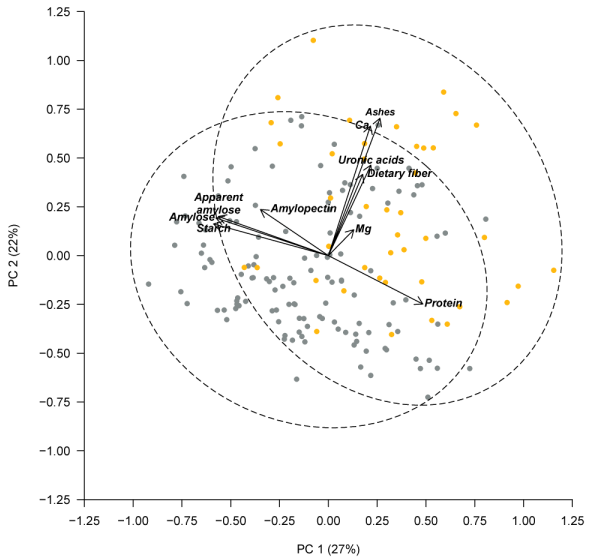

FIGURE 3 | Principal component analysis based on chemical composition of the seed coat and cotyledon, considering the gene pool classification reported by PérezVega et al. (2009). Accessions with Middle American origin are represented by yellow symbols and accessions with Andean classification are represented by gray symbols. The dashed lines represent the 95% confidence ellipses.

examining smaller collections, especially those done within a single variety or a few varieties, which found genetic correlations between chemical components and sensory traits (Casañas et al., 2006; Rivera et al., 2015). Therefore, in a large collection with greater variability, these effects are understandably diluted. Moreover, considering the important GxE effects on quantitative traits, and more specifically on traits related to chemical composition (Shellie and Hosfield, 1991; Moraghan and Grafton, 2001; Florez et al., 2009) and sensory profile (Romero del Castillo et al., 2008), significant correlations should be validated in further studies considering different agroclimatic conditions. For instance, in our case, we have performed the experiment in a field with high content of Ca and low content of P. Soil composition influences the chemical composition of common beans (Sameni et al., 1980; Moraghan and Grafton, 2001). Thus new experiments should be conducted in sites with highly different edaphologic characteristics to complement this study.

Seed coat color in common beans is controlled by several loci that act independently or in an epistatic manner to affect the color and pattern (Moghaddam et al., 2014). The P locus is known as the core factor for all seed coat color genotypes. The presence of its recessive allele results in white seeds and flowers due to its epistatic effect on the expression of the other color and pattern genes (Bassett, 2007). The background color and different patterns of marking on the seed coat are caused by the accumulation of anthocyanin and phenolic substances, which influence nutritional value and are coded by various gene systems (Beninger et al., 1999, 2000; Caldas and Blair, 2009). Our results show that there are significant differences in seed coat composition (ash, Ca, dietary fiber, Mg, and uronic acid) between white and colored seeds, but no differences for cotyledon composition (except for starch) (**Table 3** and **Figure 2**). Although the negative correlation between starch and protein ($r = -0.60$, $p < 0.001$), which was confirmed within both groups (white seeds, $r = -0.53$, $p < 0.01$; colored seeds, $r = -0.60$, $p < 0.001$), the significant differences between color groups for the starch are not reflected in the protein content. Mean values for protein content in colored accessions was lower with respect to white seeded, but these differences were not statically supported, mainly because the variation within groups was very high for this trait. In contrast, other authors found greater amounts of protein and other nutrients in the whole seeds of black beans compared to lighter varieties (Silva et al., 2012; Hacisalihoglu and Settles, 2013). Our results concur with the results reported by Casañas et al. (2013), who reported five QTL associated with ash, Ca, dietary fiber, Mg, and uronic acid content, which were mapped in the region of the P gene.

Common beans were independently domesticated in the Andean and Middle American areas. Many authors have studied this phenomenon and attributed differentiated characteristics to beans originating from each area (Gepts and Debouck, 1991; Singh et al., 1991; Schmutz et al., 2014), but the differences for chemical composition have been scarcely studied. Research performed by PérezVega et al. (2009), comparing molecular markers and phaseolins in the Spanish core collection of common bean, enabled the classification of accessions into the Middle American or Andean gene pool. Following this classification, we performed an ANOVA that showed significant differences for all chemical components in the cotyledon, with the exception of amylopectin, and significant differences only for ash and Ca in the seed coat (**Table 4** and **Figure 3**). Considering that phaseolins

are the major seed storage protein (constitute over 50% of total protein in beans), and that the concentration and type of phaseolin present contribute to the classification gene pool (Gepts et al., 1986; Singh et al., 1991), it is to be expected that cotyledon components are those with the greatest differences depending on gene pool. Results obtained from our study indicate that seeds from the Spanish core collection identified as of the Middle American gene pool have a higher concentration of protein, ash, and Ca, and lower concentrations of carbohydrates. Some of these results are in accordance with those obtained by Islam et al. (2002). In their study on the CIAT core collection of beans, they looked at these and other chemical components in whole seeds from the two major gene pools (Middle American and Andean), as well as from the North Andean Group and a mixed group. Their study showed that accessions from the Middle American gene pool contained higher Ca concentrations than those from the North Andean and Andean gene pools. However, their study also reported a lower concentration of phaseolin protein in the Middle American gene pool.

CONCLUSION

In conclusion, results of this work show that the variability in the Iberian Peninsula, a second center of diversity for *P. vulgaris*, is very high, and can be used as an important source for breeding more nutritional and palatable varieties or directly by farmers. Moreover, our results point out that there are not strong correlations between the most important nutritional and sensory attributes, which is an important finding, signaling that synchronic improvement of both traits is feasible. Significant differences for nutritional composition have been identified between colored and non-colored seeds and between gene pools (Middle American and Mesoamerican), contributing to the knowledge about the diversification process of this species. NIRS models improved in this work can be an useful technology for mass phenotyping of other sources of genetic diversity within the species.

AUTHOR CONTRIBUTIONS

FC, AR, MP, and JSa planned the study. AR, AR, and JSa conducted the experiments. MP carried out the improvement of models and obtained the data. AR, FC, MP, and JC wrote the manuscript. JSi revised the article critically. All authors read and approved the final manuscript.

FUNDING

This work has been funded by the National Institute for Agronomic Research (INIA), projects RF2010-00005-C05 and RTA 2011-00076-C02-02.

REFERENCES

Aastveit, A. H., and Marum, P. (1993). Near-infrared reflectance spectroscopy: different strategies for local calibrations in analyses of forage quality. *Appl. Spectrosc.* 47, 463–469. doi: 10.1366/0003702934334912

Araújo, S. S., Beebe, S., Crespi, M., Delbreil, B., González, E. M., Gruber, V., et al. (2015). Abiotic stress responses in legumes: strategies used to cope with environmental challenges. *Crit. Rev. Plant Sci.* 34, 237–280. doi: 10.1080/07352689.2014.898450

Asensio, C., Asensio-S-Manzanera, M., Ibeas, A., and De la Rosa, L. (2010). Resistance to halo blight common bacterial blight and bacterial brown spot in Spanish common bean core collection. *Annu. Report. Bean Improv. Coop.* 53, 101–111.

Barton, F. E., Shenk, J. S., Westerhaus, M. O., and Funk, D. B. (2000). The development of near infrared wheat quality models by locally weighted regressions. *J. Near Infrared Spectrosc.* 8, 201–208. doi: 10.1255/jnirs.280

Bassett, M. J. (2007). Genetics of seed coat color and pattern in common bean. *Plant Breed. Rev.* 28, 239–315. doi: 10.1002/9780470168028.ch8

Beninger, C. W., Hosfield, G. L., and Bassett, M. J. (1999). Flavonoid composition of three genotypes of dry bean (*Phaseolus vulgaris* L.) differing in seedcoat color. *J. Am. Soc. Hort. Sci.* 124, 514–518.

Beninger, C. W., Hosfield, G. L., Bassett, M. J., and Owens, S. (2000). Chemical and morphological expression of the B and Asp seedcoat genes in *Phaseolus vulgaris* L. *J. Am. Soc. Hortic. Sci.* 125, 52–58.

Caldas, G. V., and Blair, M. W. (2009). Inheritance of seed condensed tannins and their relationship with seed-coat color and pattern genes in common bean (*Phaseolus vulgaris* L.). *Theor. Appl. Genet.* 119, 131–142. doi: 10.1007/s00122-009-1023-4

Casañas, F., Pérez-Vega, E., Almirall, A., Plans, M., Sabaté, J., and Ferreira, J. J. (2013). Mapping of QTL associated with seed chemical content in a RIL population of common bean (*Phaseolus vulgaris* L.). *Euphytica* 192, 279–288. doi: 10.1007/s10681-013-0880-8

Casañas, F., Pujolà, M., Bosch, L., Sánchez, E., and Nuez, F. (2002). Chemical basis for low sensory perception of Ganxet bean (*Phaseolus vulgaris* L.) seed coat. *J. Sci. Food Agric.* 82, 1282–1286. doi: 10.1002/jsfa.1193

Casañas, F., Pujolà, M., Romero, del Castillo, R., Almiralll, A., Sanchez, E., et al. (2006). Variability in some texture characteristics and chemical composition of common beans (*Phaseolus vulgaris* L.). *J. Sci. Food Agric.* 86, 2445–2449. doi: 10.1002/jsfa.2638

Coelho, C. M. M., de Mattos Bellato, C., Santos, J. C. P., Ortega, E. M. M., and Tsai, S. M. (2007). Effect of phytate and storage conditions on the development of the 'hard-to-cook' phenomenon in common beans. *J. Sci. Food Agric.* 87, 1237–1243. doi: 10.1002/jsfa.2822

De la Rosa, L., Lázaro, A., and Varela, F. (2000). *Racionalización de la colección española de Phaseolus vulgaris L. II Seminario de judía de la Península Ibérica*. Villaviciosa: Asoc Esp Leguminosas, 55–62.

De Ron, A. M., Sparvoli, F., Pueyo, J. J., and Bazile, D. (2017). Editorial: protein crops: food and feed for the future. *Front. Plant Sci.* 8:105. doi: 10.3389/fpls.2017.00105

Ekmekcioglu, C., Wallner, P., Kundi, M., Weisz, U., Haas, W., and Hutter, H. P. (2018). Red meat, diseases, and healthy alternatives: a critical review. *Crit. Rev. Food Sci. Nutr.* 58, 247–261. doi: 10.1080/10408398.2016.1158148

FAO (2010). *Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture*. Available at: http://www.fao.org/docrep/013/i1500e/i1500e00.htm

FAO (2016). *International Year of Pulses*. Available at: http://www.fao.org/pulses-2016/faq/en/

FAOSTAT (2018). *Food and Agriculture Data*. Rome: FAOSTAT.

Florez, A., Pujolà, M., Valero, J., Centelles, E., Almirall, A., and Casañas, F. (2009). Genetic and environmental effects on chemical composition related to sensory traits in common beans (*Phaseolus vulgaris* L.). *Food Chem.* 113, 950–956. doi: 10.1016/j.foodchem.2008.08.036

Gepts, P., and Debouck, D. (1991). "Origin, domestication, and evolution of the common bean (*Phaseolus vulgaris* L.)," in *Common Beans: Research for Crop*

Improvement, eds A. van Schoonhoven and O. Voysest (Wallingford: CAB International-CIAT), 7–53.

Gepts, P., Osborn, T., Rashka, K., and Bliss, F. (1986). Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*): evidence for multiple centers of domestication. *Econ. Bot.* 40, 451–468. doi: 10.1007/BF02859659

Gonzales, J. F., Barnard, N. D., Jenkins, D. J. A., Lanou, A. J., Davis, B., Saxe, G., et al. (2014). Applying the precautionary principle to nutrition and cancer. *J. Am. Coll. Nutr.* 33, 239–246. doi: 10.1080/07315724.2013.866527

Gonzalez, A. D., Frostell, B., and Karlsson-Kanyama, A. (2011). Protein efficiency per unit energy and per unit greenhouse gas emissions: potential contribution of diet choices to climate change mitigation. *Food Policy* 36, 562–570. doi: 10.1016/j.foodpol.2011.07.003

Hacisalihoglu, G., Larbit, B., and Settles, A. M. (2010). Near-infrared reflectance spectroscopy predicts protein, starch and seed weight in intact seeds of common bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* 58, 502–506. doi: 10.1021/jf9019294

Hacisalihoglu, G., and Settles, A. M. (2013). Natural variation in seed composition of 91 common bean genotypes and their possible association with seed coat color. *J. Plant Nutr.* 36, 772–780. doi: 10.1080/01904167.2012.754041

Hayat, I., Ahmad, A., Masud, T., Ahmed, A., and Bashir, S. (2014). Nutritional and health perspectives of beans (*Phaseolus vulgaris* L.): an overview. *Crit. Rev. Food Sci. Nutr.* 54, 580–592. doi: 10.1080/10408398.2011.596639

Hodgkin, T., Rao, V., Cibrian-Jaramillo, A., and Gaiji, S. (2003). The use of ex situ conserved plant genetic resources. *Plant Genet. Resour.* 1, 19–29. doi: 10.1079/PGR200313

Ishitani, M., Rao, I., Wenz, P., Beebe, S., and Tohme, J. (2004). Integration of genomics approach with traditional breeding towards improving abiotic stress adaptation: drought and aluminium toxicity as case studies. *Field Crop Res.* 90, 35–45. doi: 10.1016/j.fcr.2004.07.004

Islam, F. M. S., Basford, K. E., Jara, C., Redden, R. J., and Beebe, S. (2002). Seed compositional and disease resistance differences among gene pools in cultivated common bean. *Genet. Resour. Crop. Evol.* 49, 285–293. doi: 10.1023/A:1015510428026

Kelly, J. D., Kolkman, J. M., and Schneider, K. (1998). Breeding for yield in dry bean (*Phaseolus vulgaris* L.). *Euphytica* 102, 343–356. doi: 10.1023/A:1018392901978

Langridge, P., and Fleury, D. (2011). Making the most of 'omics' for crop breeding. *Trends Biotechnol.* 29, 33–40. doi: 10.1016/j.tibtech.2010.09.006

Leterme, P. (2002). Recommendations by health organizations for pulse consumption. *Br. J. Nutr.* 88, 239–242. doi: 10.1079/BJN2002712

Martens, H., and Naes, T. (1989). *Multivariate Calibration*. New York, NY: John Wiley & Son.

McDermott, J., and Wyatt, A. J. (2017). The role of pulses in sustainable and healthy food systems. *Ann. N. Y. Acad. Sci.* 1392, 30–42. doi: 10.1111/nyas.13319

Mendiburu, F. (2017). *Agricolae: Statistical Procedures for Agricultural Research. R package version* 1.2-8. Avilable at: https://CRAN.R-project.org/package=agricolae

Micha, R., Wallace, S. K., and Mozaffarian, D. (2010). Red and processed meat consumption and risk of incident coronary heart disease stroke, and diabetes mellitus: an updated review and meta-analysis. *Circulation* 121, 2271–2283. doi: 10.1161/CIRCULATIONAHA.109.924977

Michael, H., Tinashe, C., Ruaraidh, S. H., Brad, K., Emily, M., Eric, W., et al. (2018). Plant genetic resources for food and agriculture: opportunities and challenges emerging from the science and information technology revolution. *New Phytol.* 217, 1407–1419. doi: 10.1111/nph.14993

Miklas, P. N., Kelly, J. D., Beebe, S. E., and Blair, M. W. (2005). Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. *Euphytica* 147, 105–131. doi: 10.1007/s10681-006-4600-5

Mkanda, A. V., Minnaar, A., and de Kock, H. L. (2007). Relating consumer preferences to sensory and physicochemical properties of dry beans (*Phaseolus vulgaris*). *J. Sci. Food Agric.* 87, 2868–2879. doi: 10.1002/jsfa.3046

Moghaddam, S. M., Stonehouse, R., Lee, R., Mamidi, S., Bello, M., Miklas, P., et al. (2014). Molecular genetic analysis of the *Phaseolus vulgaris* P locus. *Annu. Rep. Bean Improv. Coop.* 57, 15–16.

Moraghan, J. T., Etchevers, J. D., and Padilla, J. (2006). Contrasting accumulations of calcium and magnesium in seed coats and embryos of common bean and soybean. *Food Chem.* 95, 554–561. doi: 10.1016/j.foodchem.2004.10.060

Moraghan, J. T., and Grafton, K. (2001). Genetic diversity and mineral composition of common bean seed. 2001. *J. Sci. Food Agric.* 81, 404–408. doi: 10.1002/1097-0010(200103)81:4<404::AID-JSFA822>3.0.CO;2-H

Murdoch, D., and Chow, E. D. (2007). *Ellipse: Functions for Drawing Ellipses and Ellipse like Confidence Regions. R package version 0.4.1*. Avilable at: https://cran.r-project.org/package=ellipse

Naes, T., Isaksson, T., and Kowalski, B. (1990). Locally weighted regression and scatter correction for near-infrared reflectance data. *Anal. Chem.* 62, 664–673. doi: 10.1021/ac00206a003

Nicolaï, B. M., Beullens, K., Bobelyn, E., Peirs, A., Sacys, W., Theoron, K. I., et al. (2007). Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: a review. *Postharvest Biol. Technol.* 46, 99–118. doi: 10.1016/j.postharvbio.2007.06.024

Pan, A., Sun, Q., Bernstein, A. M., Manson, J. A. E., Willett, W. C., and Hu, F. B. (2013). Changes in red meat consumption and subsequent risk of type 2 diabetes mellitus: three cohorts of US men and women. *JAMA Intern. Med.* 173, 1328–1335. doi: 10.1001/jamainternmed.2013.6633

Paredes, M. C., Becerra, V., and Tay, J. U. (2009). Inorganic nutritional composition of common bean (*Phaseolus vulgaris* L.) genotypes race Chile. *Chil. J. Agric. Res.* 69, 486–495. doi: 10.4067/S0718-58392009000400002

Parlak, Y., and Güzeler, N. (2016). Nuclear magnetic resonance spectroscopy applications in foods. *Curr. Res. Nutr. Food Sci.* 4, 161–168. doi: 10.12944/CRNFSJ.4.Special-Issue-October.22

Pascual, A., Campa, A., Pérez-Vega, E., Giraldez, R., Miklas, P. N., and Ferreira, J. J. (2010). Screening common bean for resistance to four *Sclerotinia sclerotiorum* isolates collected in northern Spain. *Plant Dis.* 94, 885–890. doi: 10.1094/PDIS-94-7-0885

Pérez-Vega, E., Campa, A., de la Rosa, L., Giraldez, R., and Ferreira, J. J. (2006). Evaluation for resistance to anthracnose of a core collection established from the CRF-INIA common bean collection. *Annu. Rep. Bean Improv. Coop.* 49, 201–202.

Pérez-Vega, E., Campa, A., de la Rosa, L., Giraldez, R., and Ferreira, J. J. (2009). Genetic diversity in a core collection established from the main Spanish bean genbank. *Crop Sci.* 49, 1372–1386. doi: 10.2135/cropsci2008.07.0409

Pimentel, D., and Pimentel, M. (2003). Sustainability of meat-based and plant-based diets and the environment. *Am. J. Clin. Nutr.* 78, 660S–663S. doi: 10.1093/ajcn/78.3.660S

Pinheiro, C., Baeta, J. P., Pereira, A. M., Domingues, H., and Ricardo, C. P. (2010). Diversity of seed mineral composition of *Phaseolus vulgaris* L. germplasm. *J. Food Comp. Anal.* 23, 319–325. doi: 10.1016/j.jfca.2010.01.005

Plans, M., Simó, J., Casañas, F., Romero, del Castillo, R., Rodriguez-Saona, L. E., et al. (2014). Estimating sensory properties of common beans (*Phaseolus vulgaris* L.) by near infrared spectroscopy. *Food Res. Int.* 56, 55–62. doi: 10.1016/j.foodres.2013.12.003

Plans, M., Simó, J., Casañas, F., and Sabaté, J. (2012). Near-infrared spectroscopy analysis of seed coats of common beans (*Phaseolus vulgaris* L.): a potential tool for breeding and quality evaluation. *J. Agric. Food Chem.* 60, 706–712. doi: 10.1021/jf204110k

Plans, M., Simó, J., Casañas, F., Sabaté, J., and Rodriguez-Saona, J. L. (2013). Characterization of common beans (*Phaseolus vulgaris* L.) by infrared spectroscopy: comparison of MIR, FT-NIR and dispersive NIR using portable and benchtop instruments. *Food Res. Int.* 54, 1643–1651. doi: 10.1016/j.foodres.2013.09.003

Prada, D. (2009). Molecular population genetics and agronomic alleles in seed banks: searching for a needle in a haystack? *J. Exp. Bot.* 60, 2541–2552. doi: 10.1093/Jxb/Erp130

R Core Team (2017). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Ramanatha Rao, V. R., and Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell Tissue Organ Cult.* 68, 1–19. doi: 10.1023/A:1013359015812

Ramirez-Lopez, L., and Stevens, A. (2016). *Resemble: Regression and Similarity Evaluation for Memory-Based Learning in Spectral Chemometrics. R package version* 1.2.2. Available at: https://CRAN.R-project.org/package=resemble

Rivera, A., Casquero, P., Mayo, S., Almirall, A., Plans, M., Simó, J., et al. (2016). Culinary and sensory traits diversity in the Spanish Core Collection of common beans (*Phaseolus vulgaris* L.). *Span. J. Agric. Res.* 14:e0701. doi: 10.5424/sjar/2016141-7726

Rivera, A., Roselló, S., and Casañas, F. (2015). Seed curvature as a useful marker to transfer morphologic, agronomic, chemical and sensory traits from Ganxet common bean (*Phaseolus vulgaris* L.). *Sci. Hortic.* 197, 476–482. doi: 10.1016/j.scienta.2015.10.006

Romero del Castillo, R., Almirall, A., Valero, J., and Casañas, F. (2008). Protected designation of origin in beans (*Phaseolus vulgaris* L.): towards an objective approach based on sensory and agromorphological properties. *J. Sci. Food Agric.* 88, 1954–1962. doi: 10.1002/jsfa.3303

Romero del Castillo, R., Costell, E., Plans, M., Simó, J., and Casañas, F. (2012). A standardized method of preparing common beans (*Phaseolus vulgaris* L.) for sensory analysis. *J. Sens. Stud.* 27, 188–195. doi: 10.1111/j.1745-459X.2012.00381

Sameni, A. M., Maftoun, M., Bassiri, A., and Sepaskhah, A. R. (1980). Growth and chemical composition of dry beans as affected by soil salinity and N fertilization. *Plant Soil* 54, 217–222. doi: 10.1007/BF02181847

Santalla, M., de Ron, A. M., and Voysest, O. (2001). "European bean market classes," in *Catalogue of Bean Genetic Resources*, eds M. Amurrio, M. Santalla, and A. M. de Ron (Corunha: Fundación Pedro Barrie de la Maza), 79–94.

Santalla, M., Rodiño, A., and de Ron, A. M. (2002). Allozyme evidence supporting southwestern Europe as a secondary center of genetic diversity for common bean. *Theor. Appl. Genet.* 104, 934–994. doi: 10.1007/s00122-001-0844-6

Sathe, S. K., Desphande, S. S., Salunkhe, D. K., and Rackis, J. J. (1984a). Dry beans of phaseolus. A review. Part 1. Chemical composition: proteins. *Crit. Rev. Food Sci.* 20, 1–46. doi: 10.1080/10408398409527382

Sathe, S. K., Desphande, S. S., Salunkhe, D. K., and Rackis, J. J. (1984b). Dry beans of phaseolus. A review. Part 2. Chemical composition: carbohydrates, fiber, minerals, vitamins and lipids. *Crit. Rev. Food Sci.* 21, 41–93. doi: 10.1080/10408398409527396

Schmutz, J., McClean, P. E., Mamidi, S., Wu, G. A., Cannon, S. B., Grimwood, J., et al. (2014). A reference genome for common bean and genome-wide analysis of dual domestications. *Nat. Genet.* 46, 707–713. doi: 10.1038/ng.3008

Shellie, K. C., and Hosfield, G. L. (1991). Genotype x environmental effects on food quality of common bean: resource efficient testing procedures. *J. Am. Soc. Hortic. Sci.* 116, 732–736.

Shenk, J. S., and Westerhaus, M. O. (1991). Population definition, sample selection and calibration procedures for near infrared reflectance spectroscopy. *Crop Sci.* 31, 469–474. doi: 10.2135/cropsci1991.0011183X003100020049x

Shenk, J. S., Westerhaus, M. O., and Berzaghi, P. (1997). Investigation of a LOCAL calibration procedure for near infrared instruments. *J. Near Infrared Spectrosc.* 5, 223–232. doi: 10.1255/jnirs.115

Silva, C. A., Abreu, A. F. B., Ramalho, M. A. P., and Maia, L. G. S. (2012). Chemical composition as related to seed color of common bean. *Crop Breed. Appl. Biotechnol.* 12, 132–137. doi: 10.1590/S1984-70332012000200006

Singh, S., Singh, H. D., and Sikka, K. C. (1968). Distribution of nutrients in the anatomical parts of common Indian pulses. *Cereal Chem.* 45, 13–18.

Singh, S. P. (1999). "Integrated genetic improvement," in *Common Bean Improvement in the Twenty-First Century Developments in Plant Breeding*, Vol. 7, ed. S. P. Singh (Dordrecht: Springer), 133–165. doi: 10.1007/978-94-015-9211-6

Singh, S. P., Gepts, P., and Debouck, D. G. (1991). Races of common bean (*Phaseolus vulgaris*, Fabaceae). *Econ. Bot.* 45, 379–396. doi: 10.1007/BF02887079

Singh, S. P., and Schwartz, H. (2009). Breeding common bean for resistance to diseases: a review. *Crop Sci.* 50, 2099–2223.

Sinnaeve, G., Dardenne, P., and Agneessens, R. (1994). Global or local? A choice for NIR calibrations in analyses of forage quality. *J. Near Infrared Spectrosc.* 2, 163–175. doi: 10.1255/jnirs.43

Smýkal, P., Coyne, C. J., Ambrose, M. J., Maxted, N., Schaefer, H., Blair, M. W., et al. (2015). Legume crops phylogeny and genetic diversity for science and breeding. *Crit. Rev. Plant Sci.* 34, 43–104. doi: 10.1080/07352689.2014.897904

Stacklies, W., Redestig, H., Scholz, M., Walther, D., and Selbig, J. (2007). PcaMethods–a bioconductor package providing PCA methods for incomplete data. *Bioinformatics* 23, 1164–1167. doi: 10.1093/bioinformatics/btm069

Tanksley, S. D., and McCouch, S. R. (1997). Seed banks and molecular maps: unlocking genetic potential from the wild. *Science* 277, 1063–1066. doi: 10.1126/science.277.5329.1063

van Hintum, T. J., Brown, A. H. D., Spillane, C., and Hodkin, T. (2000). *Core Collections of Plant Genetic Resources*. Rome: International Plant Genetic Resources Institute.

Vargas-Torres, A., Osorio-Díaz, P., Tovar, J., Paredes-López, O., Ruales, J., and Bello-Pérez, L. A. (2004). Chemical composition, starch bioavailability and indigestible fraction of common beans (*Phaseolus vulgaris* L.). *Starke* 56, 74–78. doi: 10.1002/star.200300205

Vaz Patto, M. C., Amarowicz, R., Aryee, A. N. A., Boye, J. I., Chung, H. J., Martín-Cabrejas, M. A., et al. (2015). Achievements and challenges in improving the nutritional quality of Food legumes. *Crit. Rev. Plant Sci.* 34, 105–143. doi: 10.1080/07352689.2014.897907

Wang, C., Hu, S., Gardner, C., and Lübberstedt, T. (2017). Emerging avenues for utilization of exotic germplasm. *Trends Plant Sci.* 22, 624–637. doi: 10.1016/j.tplants.2017.04.002

WHO (2015). *World Health Organization*. Available at: http://www.who.int/mediacentre/factsheets/fs394/en/

Wiesinger, J. A., Cichy, K. A., Glahn, R. P., Grusak, M. A., Brick, M. A., Thompson, H. J., et al. (2016). Demonstrating a nutritional advantage to the fast-cooking dry bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* 64, 8592–8603. doi: 10.1021/acs.jafc.6b03100

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 October 2018
doi: 10.3389/fpls.2018.01460

Genetic Characterization of the Apple Germplasm Collection in Central Italy: The Value of Local Varieties

Gianpiero Marconi[1†], Nicoletta Ferradini[1†], Luigi Russi[1†], Luciano Concezzi[2], Fabio Veronesi[1] and Emidio Albertini[1]*

[1] Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università degli Studi di Perugia, Perugia, Italy, [2] 3a Parco Tecnologico Agroalimentare dell'Umbria, Perugia, Italy

In the last 50 years, intensive farming systems have been boosted by modern agricultural techniques and newly bred cultivars. The massive use of few and related cultivars has dramatically reduced the apple genetic diversity of local varieties, confined to marginal areas. In Central Italy a limited spread of intensive fruit orchards has made it possible to preserve much of the local genetic diversity, but at the same time the coexistence of both modern and ancient varieties has generated some confusion. The characterization and clarification of possible synonyms, homonyms, and/or labeling errors in old local genetic resources is an issue in the conservation and management of living collections. 175 accessions provided by 10 apple collections, mainly local varieties, some of unknown origin, and well-known modern and ancient varieties, were studied by using 19 SSRs, analyzed by STRUCTURE, Ward's clustering and parentage analysis. We were able to identify 25 duplicates, 9 synonyms, and 9 homonyms. As many as 37 unknown accession were assigned to well known local or commercial varieties. Polyploids made up 20%. Some markers were found to be significantly correlated with morphological traits and the *loci* associated with the fruit over color were related to QTLs for resistance to biotic stresses, aroma compounds, stiffness, and acidity. In conclusion the gene pool of Central Italy seems to be rather consistent and highly differentiated compared with other European studies (F_{ST} = 0.147). The importance of safeguarding this diversity and the impact on the management of the germplasm living collection is discussed.

Keywords: *Malus* × *domestica*, SSR markers, local varieties, genetic resources, germplasm collection

OPEN ACCESS

Edited by:
Spyridon Alexandros Petropoulos, University of Thessaly, Greece

Reviewed by:
Aureliano Bombarely, Virginia Tech, United States
Gerhard Buck-Sorlin, Agrocampus Ouest, France
Nicolò Cultrera, Istituto di Bioscienze e Biorisorse (IBBR), Italy

***Correspondence:**
Emidio Albertini
emidio.albertini@unipg.it

† These authors have contributed equally to this work as first authors

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *02 July 2018*
Accepted: *12 September 2018*
Published: *10 October 2018*

Citation:
Marconi G, Ferradini N, Russi L, Concezzi L, Veronesi F and Albertini E (2018) Genetic Characterization of the Apple Germplasm Collection in Central Italy: The Value of Local Varieties. Front. Plant Sci. 9:1460. doi: 10.3389/fpls.2018.01460

INTRODUCTION

Apple (*Malus* × *domestica* Borkh., family Rosaceae, tribe Pyreae, $2n = 2x = 34$) is one of the most ancient and widespread fruit crops in temperate regions. Almost certainly the domesticated apple is the result of a long evolutionary process extending over thousands of years, and it seems several species have contributed to its gene pool. On the basis of genetic data, of fruit and tree morphology, the wild Asian species *M. sieversii* M. Roem is actually considered the main contributor to the *Malus* × *domestica* gene pool, and the Tian Shan Mountains (Central Asia) the center of origin

(Velasco et al., 2010; Cornille et al., 2014). Furthermore, hybridizations with other wild apple species present along the Silk Route, such as *M. baccata* (L.) Borkh. in Siberia, *M. orientalis* Uglitz. in Caucasus and *M. silvestris* (L.) Miller in Europe, have produced the diversity currently present in the domesticated apple (Vavilov, 1926; Harris et al., 2002; Cornille et al., 2012, 2014; Gross et al., 2014). Despite high genetic variability, the thousands of cultivars distributed throughout the world and the world-wide breeding programs, mainly based on organoleptic traits, aesthetic standards and disease resistance, the size of the genetic resources used by breeders has been limited and reduced to a few varieties such as 'Cox's Orange Pippin,' 'Golden Delicious,' 'Jonathan,' 'Red Delicious,' and 'McIntosh' (Noiton and Alspach, 1996). As a result, apple cultivation today is limited to closely related cultivars, and four of them, namely Golden Delicious, Gala, Red Delicious, and Idared, account for 48% of global production. Actually, the most important variety is Golden Delicious with 2.546 million metric tons, followed, by Gala with 1.331 million metric tons and Idared with 1.111 million metric tons (Food and Agricultural Organization of the United Nation [FAO], 2016; WAPA, 2017). This massive use of limited and related cultivars, combined with vegetative practices based on cuttings and grafting, has dramatically reduced apple genetic diversity and, hence, many interesting and well adapted traditional and local varieties, considered obsolete, were no longer cultivated and have been partly lost (Hammer et al., 2003).

Similar trends occurred in Italy, which with its 2.5 million t represents the fifth apple producer in the World and the second in Europe (Food and Agricultural Organization of the United Nation [FAO], 2016). Apple production is mainly concentrated in the North of Italy, in particular in Trentino Alto Adige, which with its 1.7 million t represents 67% of total Italian production, followed by Veneto (11%), Piemonte (6%), and Emilia-Romagna (6%). In these regions, as well as in the World and in other European Countries, production is based mainly on intensive orchards with few commercial varieties: Golden Delicious, Gala, Red Delicious, Fuji, and Granny Smith (Assomela-CSO, 2017). In Center-South Italy intensive apple orchards are appreciably present only in Campania (3% of Italian production). Historically these areas have never been inclined to intensive cultivation of fruit in general and apples in particular, even if in the 1920s there were unsuccessful attempts to introduce improved varieties in intensive orchards (Albertini et al., 2015). In these regions, apart from few family-run orchards, fruit production was, and currently remains, mainly directed toward self-consumption and local markets. Therefore, in Central Italy the almost complete absence of intensive apple cultivation allowed for the preservation of much of the existing genetic diversity even if the limited coexistence between modern and local varieties and the evolution of the farming systems since the 1950s, has led to the disappearance of several interesting and well adapted ancient varieties. Many of these varieties, although of low productivity, were relatively stable under extreme environmental conditions, and their high genetic variability guaranteed reliable harvesting for local communities in the past (Albertini et al., 2015). Over time, some of the modern, introduced varieties mixed with the autochthones, increasing the panorama of choice on the one hand, but generating some confusion regarding local genetic resources and their correct denomination on the other. Consequently, the need for characterization and clarification of possible synonyms, homonyms, and/or labeling errors in these old and local genetic resources is a fundamental and necessary step for the conservation and management of living collections. Indeed, the genetic variability and allelic diversity present in these old accessions could be of extreme interest in terms of response to selection in adaptation toward a changing environment (Caballero and García-Dorado, 2013). Therefore, even though such varieties are characterized by low fruit quality and yield, their allelic diversity could be essential for crop improvement, providing the presence of interesting traits for the development of new varieties.

In this scenario, molecular markers can provide a valid tool to assess genetic diversity, uncovering duplicates or possible synonymies and/or homonymies, and helping the management of plant genetic resources. Several studies based on molecular markers have assessed the diversity in *Malus*, including modern cultivars and old, local germplasm accessions (Pereira-Lorenzo et al., 2007, 2008, 2017; Gharghani et al., 2009; Gasi et al., 2010; van Treuren et al., 2010; Gross et al., 2012; Urrestarazu et al., 2012; Gao et al., 2015; Liang et al., 2015; Lassois et al., 2016; Vanderzande et al., 2017). For these purposes microsatellites (SSRs) are considered the marker of choice due their high polymorphism and co-dominant inheritance, widely used to asses the genetic diversity at population and individual level in many plant core collections (Blair et al., 2009; Zhang et al., 2011; Patzak et al., 2012; Emanuelli et al., 2013; Urrestarazu et al., 2016; Mousavi et al., 2017). To date several hundred SSR markers have been developed and mapped in apples by Guilford et al. (1997), Gianfranceschi et al. (1998), Liebhard et al. (2002), Vinatzer et al. (2004), Silfverberg-Dilworth et al. (2006), Han and Korban (2008), and Celton et al. (2009). Many of these mapped SSR are linked to QTL with interesting agronomical, morphological, and organoleptic traits and could be used as molecular tools for marker assisted selection (MAS) in plant breeding programs (Gianfranceschi et al., 1998; Gygax et al., 2004; Kunihisa et al., 2014; Sun et al., 2014; Liu et al., 2016). Microsatellites have been also widely used in apples to asses the genetic diversity in core collections (Yun et al., 2015; Lassois et al., 2016; Urrestarazu et al., 2016), cultivar characterization (Goulão and Oliveira, 2001; Patzak et al., 2012; Pérez-Romero et al., 2015), and parentage analysis (Kitahara et al., 2005; Moriya et al., 2011; Lassois et al., 2016).

The present research aims to understand the relationship between local accessions in central Italy with old and new varieties, as well as investigate for synonyms/homonyms for a better management of conservation and propagation of genetic resources.

MATERIALS AND METHODS

Plant Material

One hundred and seventy five accessions of *Malus* × *domestica*, mostly Italian, were included in this study and were provided by

several collections: the National Center of Fruit Tree Germplasm (CREA, coded c01), Parco Tecnologico Agroalimentare dell'Umbria (3A-PTA, coded c02 and c03 as coming from two living collections), the Department of Agriculture, Forestry and Food Science of the University of Torino (DISAFA, c04), the Archeologia Arborea private collection (c05), the Department of Agricultural, Food and Environmental Sciences of the Polytechnic University of Marche (D3A, c06), Malva Rinaldi School in Torino (c07), the Department of Agricultural Science, University of Bologna (DipSA, c08), the Giardino Armonico of Bevagna private collection (c09) and Azienda Ortofrutticola Sett'Olmi, Perugia (c10). Details are reported in **Table 1**. Many of the 175 accessions were well documented by reliable historical sources. Those lacking of several reliable information were coded as Unknown. Therefore, based on the initial information, the 175 accessions used in this study were classified into 17 commercial varieties (CV) used as control, 99 local varieties (LV), and 59 unknown accessions (UA).

Microsatellite Amplification

Total genomic DNA was purified from young leaves using the DNeasy 96 Plant Kit (Qiagen) according to manufacturer's protocol. Twenty one apple SSR primer pairs (Liebhard et al., 2002; Vinatzer et al., 2004; Silfverberg-Dilworth et al., 2005) distributed over the 17 apple linkage groups were used (**Table 2**). Primer sequence and allele range for validated *loci* were analyzed by Multiplex Manager (Holleley and Geerts, 2009) to determine the best sets of *loci* to combine in a multiplex protocol. Multiplex Manager was used with the option of grouping all validated *loci* within the minimum number of PCRs avoiding allele range overlap and primer interactions.

PCRs were carried out in a final volume of 25 μl using 1 × Type-it Microsatellite PCR Master Mix (Qiagen), 0.2 μM of each fluorescent forward primer labeled with 6-FAM or ROX dyes (Sigma) and reverse unlabeled primer and 20 ng of template DNA. All amplifications were performed in a GeneAmpPCRSystem 9700 (Applied Biosystems, United States) consisting of a denaturing step of 5 min at 95°C followed by 30 cycles of 95°C for 30 s, 57°C for 90 s and 72°C for 30 s, and a final elongation step of 30 min at 60°C.

PCR products were separated and analyzed on a 3130 XL DNA Analyzer (Applied Biosystems). The size of the amplified products was determined on internal standard DNA (GeneScan 500 Liz, Thermo) and the scorable peaks were assigned by GeneMapper software v.4.0 (Applied Biosystems).

Morphological Characterization

Phenological and morphological traits of interest were scored as follows: time of eating maturity (1 = early summer, 3 = late summer-early autumn, 5 = autumn, 7 = winter); fruit shape (1 = cylindrical waisted, 2 = conic, 3 = ovoid, 4 = cylindrical, 5 = ellipsoid, 6 = globose, 7 = obloid); fruit ground color (1 = green, 3 = yellow, 5 = red); hue over color of fruit (1 = absent, 3 = yellow, 5 = orange, 7 = pink, 9 = red); fruit rustiness (1 = absent, 9 present), pulp color (1 = white, 3 = cream, 5 = yellow, 7 = red). The scores were assigned by 3 operators who exchanged opinions before eventually providing individual

TABLE 1 | Collection code[1], accession name, ploidy[2] (D, diploids; P, polyploids) and status (LV, local variety; CV, commercial variety; UA, unknown) of 175 apple accessions used in the present study.

Coll. code	Accession name	Ploidy	Status
c01	001_Cerina	D	LV
c01	002_Zuccherina	D	LV
c01	003_Gelato Cola	D	LV
c01	004_Ghiacciola	P	LV
c01	005_Pom de L'oio Rosso	D	LV
c01	006_'E Santu Giuanni	D	LV
c01	007_'E Santu Giuanni Rossa	D	LV
c01	008_Roggia	D	LV
c01	009_Ruzine	D	LV
c01	010_Ruzza	D	LV
c01	011_Sona	D	LV
c02	012_Ruzza	D	LV
c02	013_San Giovanni	D	LV
c02	014_Oleosa	D	LV
c02	015_A Sonagli	D	LV
c02	016_Gelata	D	LV
c02	017_Cera	D	LV
c03	018_Roggia	D	LV
c04	019_Gris d'la composta	D	LV
c04	020_Gris canavoeit	D	LV
c04	021_San Sebastian	P	LV
c04	022_Ruggine piatta	D	LV
c04	023_Buras	P	LV
c04	024_Grigia di Torriana	D	LV
c05	025_Oliata	P	LV
c05	026_Diacciata	P	LV
c06	027_Oleata	D	LV
c06	028_Cerina	D	LV
c06	029_Gelata	D	LV
c06	030_Gelata	D	LV
c02	031_Olia	D	LV
c02	032_Casciola	P	LV
c02	033_Panaia	D	LV
c02	034_Panaia	P	LV
c02	035_Pagliaccia	P	LV
c02	036_Casciola	P	LV
c02	037_Casciola	D	LV
c07	038_Sonaja Rossa	D	LV
c07	039_Ciocarina Bianca	D	LV
c07	040_Ciocarina Rossa Dossa	D	LV
c07	041_Ciochera Rosa	D	LV
c08	042_Pum Giuan	D	CV
c08	043_San Giovanni PT	D	LV
c08	044_San Giovanni MO	P	LV
c08	045_San Giovanni BO	P	LV
c08	046_Ceres	D	LV
c02	047_Golden Delicious	D	CV
c02	048_Golden Gala	D	CV
c02	049_Amerina	D	LV
c02	050_Pianella	P	LV
c02	051_Unknown	D	UA
c02	052_Appiola Rossa	D	LV

(Continued)

TABLE 1 | Continued

Coll. code	Accession name	Ploidy	Status
c02	053_Rosa D'Amelia	D	LV
c02	054_Unknown	P	UA
c02	055_Unknown	D	UA
c02	056_Unknown	P	UA
c02	057_Bianchina	D	LV
c02	058_Coccianese	D	LV
c02	059_Coccianese	D	LV
c02	060_Unknown	D	UA
c02	061_Limoncella	D	LV
c02	062_Piattuccia	D	LV
c02	063_Stratarina	D	LV
c02	064_Conventina	D	LV
c02	065_Muso di Bue	D	LV
c02	066_Ruzza	D	LV
c02	067_Spoletina	P	LV
c02	068_Unknown	D	UA
c02	069_Unknown	P	UA
c02	070_Rossa Doglio	D	LV
c02	071_Gialla Doglio	D	LV
c02	072_Ciucca Dolcetta	D	LV
c02	073_Polsola	P	LV
c02	074_Dolcetta	D	LV
c02	075_Unknown	D	UA
c02	076_Unknown	D	UA
c02	077_Appiola Rossa	P	LV
c02	078_Appiola	D	LV
c02	079_Unknown	D	UA
c02	080_Ducale	D	LV
c02	081_Pianella	D	LV
c02	082_Rosciola	D	LV
c02	083_Unknown	P	UA
c02	084_Unknown	D	UA
c02	085_Unknown	D	UA
c02	086_Ulpia	D	LV
c02	087_Amerina	D	LV
c02	088_Saragano Rossa	D	LV
c02	089_Unknown	D	UA
c02	090_Unknown	D	UA
c02	091_Saragano Gialla	D	LV
c02	092_Unknown	D	UA
c02	093_Unknown	P	UA
c02	094_Unknown	D	UA
c02	095_Unknown	D	UA
c02	096_Unknown	D	UA
c02	097_Unknown	D	UA
c02	098_Unknown	D	UA
c02	099_Unknown	P	UA
c02	100_Unknown	D	UA
c02	101_Maggiolina	D	LV
c02	102_Unknown	P	UA
c02	103_Unknown	D	UA
c02	104_Rossa Montelupone	D	LV
c02	105_Unknown	D	UA

(Continued)

TABLE 1 | Continued

Coll. code	Accession name	Ploidy	Status
c02	106_Unknown	D	UA
c02	107_Gialla Montelupone	D	LV
c02	108_Unknown	D	UA
c02	109_Unknown	P	UA
c02	110_Unknown	D	UA
c02	111_Unknown	D	UA
c02	112_Paradisa	D	LV
c02	113_Unknown	D	UA
c02	114_Unknown	P	UA
c02	115_Coppiola	D	LV
c02	116_Unknown	P	UA
c02	117_Unknown	D	UA
c03	118_Rosa in Pietra	D	LV
c03	119_Del Castagno	D	LV
c03	120_Ciucca	D	LV
c03	121_Rosa Gentile	D	LV
c03	122_Rosa Romana	P	LV
c03	123_Polsola	D	LV
c03	124_Bianchina	P	LV
c03	125_Unknown	D	UA
c03	126_Limoncella	D	LV
c03	127_Conventina	D	LV
c03	128_Roggia	D	LV
c09	129_Durello	D	LV
c09	130_Calvilla d'Estate	D	CV
c09	131_Reinette du Canada	P	CV
c09	132_Reinette Ananas	D	CV
c09	133_Reinette de Champagne	D	CV
c09	134_Limoncina	D	CV
c09	135_Decio	D	LV
c09	136_Annurca	D	CV
c09	137_Abbondanza	D	CV
c02	138_Unknown	D	UA
c02	139_Spoletina	P	LV
c02	140_Unknown	P	UA
c02	141_Limoncella	D	LV
c02	142_Unknown	D	UA
c02	143_Unknown	D	UA
c02	144_Unknown	D	UA
c02	145_Unknown	P	UA
c02	146_Gelata	D	LV
c02	147_Rosa in Pietra	D	LV
c02	148_Unknown	D	UA
c02	149_Coccianese	D	LV
c02	150_Unknown	D	UA
c02	151_Unknown	D	UA
c02	152_Cera	D	LV
c02	153_Unknown	D	UA
c02	154_Unknown	D	UA
c02	155_Unknown	D	UA
c02	156_Statia	D	LV
c02	157_Unknown	D	UA
c02	158_Paonazza di Piubbica	D	LV

(Continued)

TABLE 1 | Continued

Coll. code	Accession name	Ploidy	Status
c02	159_Annurca	D	CV
c02	160_Unknown	D	UA
c02	161_Unknown	P	UA
c02	162_Unknown	P	UA
c02	163_Unknown	P	UA
c02	164_Unknown	D	UA
c02	165_Rossa di San Venanzo	D	CV
c02	166_Unknown	D	UA
c02	167_Unknown	D	UA
c02	168_Unknown	D	UA
c02	169_Rosona	P	LV
c10	170_Golden Clone B	D	CV
c10	171_Fuji	D	CV
c10	172_Stark Delicious	D	CV
c10	173_Unknown	D	UA
c10	174_Gold Chief (Gold Pink)	D	CV
c10	175_Cripps Pink	D	CV

[1]c01 = CREA, National Centre of Fruit Tree Germplasm; c02 = 3A-PTA, Parco Tecnologico Agroalimentare dell'Umbria, Pantalla; c03 = 3A-PTA, Casalina; c04 = DISAFA, Department of Agriculture, Forestry and Food Science, University of Torino; c05 = Archeologia Arborea, private collection; c06 = D3A, Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University; c07 = School Malva Rinaldi of Torino; c08 = DipSA, Department of Agricultural Science, University of Bologna; c09 = Giardino Armonico of Bevagna, private collection; c10 = Nursery Sett'Olmi, private collection. [2]An accession is considered polyploid when at least 3 out of 19 loci showed a third allele.

scores, which were averaged prior to statistical analysis. Data were available only for 130 accessions and were therefore used primarily to ascertain doubts (**Supplementary Table S1**).

Data Analysis

For each *locus*, common PCR artifacts leading to genotyping error were investigated. Presence of null alleles, large allele dropout and extreme stuttering was inferred by means of bootstrapping in Micro-Checker v2.2.3 (Van Oosterhout et al., 2004) based on 1000 bootstraps and 95% confidence interval. A preliminary analysis detected a deviation due the presence of null alleles for two *loci* (Hi23g02 and Hi03g02) which were therefore discarded. The statistical analysis was then based on the remaining 19 *loci* (**Supplementary Table S2**).

The statistical analysis of the SSR data matrix was carried out by Genodive (Meirmans and Van Tienderen, 2004) and SPAGeDi1.2 (Hardy and Vekemans, 2002). Both software packages are able to analyze data files containing diploid and polyploid accessions together. The analysis included: the detection of the number of allele (Na) per *locus*, the effective number of alleles (Ne), the percentage of rare alleles (RA = allele frequency < 0.01), of the observed (H_o) and expected (H_e) heterozygosity (Nei, 1978), inbreeding coefficient F, polymorphic information content (PIC) (Botstein et al., 1980) at each *locus*, determined using the following equation:

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \left(\sum_{i=n}^{n} p_i^2\right)^2 + \sum_{i=n}^{n} p_i^4$$

The analysis also included the probability of identity (P_{ID}) (Waits et al., 2001) and the probability of identity among sibs $P_{(ID)sib}$ (Evett and Weir, 1998), calculated as follows:

$$P_{ID} = \sum p_i^4 + \sum\sum \left(2p_i p_j\right)^2$$

$$P_{(ID)sib} = 0.25 + \left(0.5\sum p_i^2\right) + \left[0.5\left(\sum p_i^2\right)^2\right] - \left(0.25\sum p_i^4\right)$$

where p_i and p_j are the frequencies of the ith and jth alleles and $i \neq j$.

Finally, the ability of each marker to discriminate two random cultivars was estimated by the Power of Discrimination ($PD = 1 - P_{ID}$) (Kloosterman et al., 1993).

In order to run a cluster analysis on diploid and polyploid accessions together, SSR data were converted to a binary data matrix by assigning "1" to the presence of a defined allele and "0" to its absence. The binary data matrix was then used to estimate a distance matrix, and the 175 accessions were clustered by Ward's hierarchical method (Ward, 1963) and validated by 1000 bootstrap replicates using PAST software (Hammer et al., 2001). The analysis revealed the presence of several identical accessions with the same genetic profile. As a consequence, these were removed and the remaining 150 were used again as binary data for a cluster analysis based on Ward's method, and the results compared with the Bayesian model-based clustering of STRUCTURE ver. 2.2.3 (Pritchard et al., 2000) using codominant data based on allele size. STRUCTURE software implements a clustering method assigning individuals and predefined populations to *K* inferred clusters, each characterized by a set of allele frequencies at Hardy-Weinberg equilibrium, based on estimates of the corresponding probabilities of membership to each group. The analyses were run on an admixture ancestral model with correlated allele frequencies, and the number of *K* clusters was determined firstly by simulating a range of *K*-values from 1 to 21 with 10 independent runs each. Since after $K = 5$ there were no other appreciable peaks, STRUCTURE was run again with twenty runs for *K*-values ranging from 1 to 11, using a burn-in and a run length of the Monte Carlo Markov Chain (MCMC) of 300,000 and 500,000 iterations for data collection. The best *K*-value was determined through the ΔK method (Evanno et al., 2005) by using the STRUCTURE HARVESTER ver. 0.6.193 website (Earl and vonHoldt, 2012). The genotypes were assigned to the groups according to their highest membership coefficient, considering a strong affinity when the assigning probability (*qI*) was ≥ 0.80 (Breton et al., 2008; Pereira-Lorenzo et al., 2008; Miranda et al., 2010; Urrestarazu et al., 2012). The software STRUCTURE is able to infer the genetic structure either in diploid or polyploid genotypes as described in using the recessive allele approach (Falush et al., 2007). The membership of each accession to the Ward's clusters and to the groups of STRUCTURE were compared and discussed.

TABLE 2 | Characteristics of the 19 SSR markers used in the study, forward and reverse sequences, repetitive motives and types (Perfect, Imperfect, Compound), and linkage group.

Locus	Forward seq. (5′– 3′)	Reverse seq. (5′- 3′)	Rep. motiv	Rep. type	Linkage group
CH05c06	atcaacagtagtggtagccgggt	attggaactctccgtattgtgc	CT	Imp	16
CH-Vf1	atcaacacgagcagcaaag	catacaaatcaaagcacaaccc	AG	Imp	1
Hi07h02	caaattggcaactgggtctg	gtttaggtggaggtgaagggatg	GT	Perf	17
CH03d12	gcccagaagcaataagtaaacc	attgctccatgcataaaggg	CT	Comp	6
CH05e03	cgaatattttcactctgactggg	caagttgttgtactgctccgac	GA	Perf	2
Hi04e04	gaccacgaagcgctgttaag	gtttcggtaattccttccatcttg	GA	Perf	16
CH02b03b	ataaggatacaaaaaccctacacag	gacatgtttggttgaaaacttg	CT	Perf	10
CH01f03b	gagaagcaaatgcaaaaccc	ctccccggctcctattctac	GA	Imp	9
CH02c09	ttatgtaccaactttgctaacctc	agaagcagcagaggaggatg	CT	Perf	15
Hi22f12	ggccctcacccagtctacatt	gtttggtgtgatggggtactttgc	CTT	Imp	5
CH01g12	cccaccaatcaaaaatcacc	tgaagtatggtggtgcgttc	CT	Imp	12
AU223657	ttctccgtccccttcaacta	caccttgaggcctctgtagc	CT	Imp	3
CH01h02	agagcttcgagcttcgtttg	atcttttggtgctcccacac	CT	Imp	9
CH01h01	gaaagacttgcagtgggagc	ggagtgggtttgagaaggtt	CT	Perf	17
CH04a12	cagcctgcaactgcacttat	atccatggtcccataaacca	CT	Imp	11
Hi03a10	ggacctgcttcccccttattc	cagggaacttgttgatgg	GA	Imp	7
CH04c07	ggccttccatgtctcagaag	cctcatgccctccactaaca	GA	Perf	14
CH01c06	ttccccatcatcgatctctc	aaactgaagccatgagggc	GA	Perf	8
CH02g01	gatgacgtcggcaggtaaag	caaccaacagctctgcaatc	CT	Perf	13
CH03d07	caaatcaatgcaaaactgtca	ggcttctggccatgatttta	CT	Perf	6
Hi23g02	ttttccaggatatactaccttcc	gtttcttcgaggtcagggtttg	CA	Perf	4

The 92 accessions whose probability (*qI*) was $\geq$ 0.80 were grouped into K = 5 subset of 7, 9, 29, 11, and 36 individuals. The goodness of fit of these 5 groups was investigated by the F statistics (F_{IS} and F_{ST}) (Weir and Cockerham, 1984), and the analysis of molecular variance (AMOVA) that estimates the fraction of the genetic variation among and within populations (Excoffier et al., 1992; Michalakis and Excoffier, 1996).

The software FaMoz (Gerber et al., 2003) was used to carry out a parentage analysis, to look for possible genetic relationships (parents) present among the entire sample of accessions and eventually confirming the results of STRUCTURE and Ward's clustering. FaMoz calculates the logarithm of the likelihood ratio, log of odds ratio (LOD score), by determining the likelihood of an individual being the parent of a given offspring, divided by the likelihood of these individuals being unrelated (Meagher and Thompson, 1986). LOD scores for any potential parentage relationship with a value greater than zero were computed, giving statistical significance to the data. Possible parents determined by LOD scores and significance thresholds were probed among the 150 accessions characterized with the set of 19 SSRs. Through 100,000 simulations with a rate of mistyping errors of 0.1% as described by Gerber et al. (2000), a LOD score threshold of 5.0 was found and used in our work (**Supplementary Figure S1**). For polyploid accessions a FaMoz control analysis was run on a 0–1 data matrix.

Moreover, in order to look for correlations between molecular data and morphological traits, a non-parametric correlation analysis (Spearman) was carried out using SAS 9.1 (Cary, NC, United States).

RESULTS

Genetic Diversity

The 19 nuclear SSRs were all polymorphic and produced scorable amplicons with a total of 278 alleles. The average number of alleles per *locus* was 14.6, ranging from 5 (Hi22f12) to 26 (CH05e03), but the number of effective alleles per *locus* was significantly lower (Ne = 5.94) (**Table 3**). With the exception of *locus* Hi22f12, all *loci* showed at least one genotype with three alleles. *Loci* CH02b03b and CH01h01 identified as many as 28 individuals with three alleles, while the other *loci* identified between 8 and 22 individuals. Even if several individuals showed one *locus* with a third allele, only those showing a third allele in at least 3 *loci* (Urrestarazu et al., 2012) were considered putative polyploids. In the present study 35 individuals (20%) were classified as polyploids (**Table 1**), as they showed a third allele from 4 up to 13 *loci*.

Rare alleles were found in 17 *loci* and were more common as the number of alleles per *locus* increased. Rare alleles ranged from 12.5% (at *locus* CH04c07 rare alleles with a frequency less than 1% were 2 out of 16) to 57% (at *locus* CH-Vf1 they were 8 out of 14). No rare allele were found at the *loci* Hi22f12 and AU223657, where the range of allele size were the lowest, 16 and 13 bp, respectively (**Table 3**).

Except for CH-Vf1 and CH01c06, all other *loci* were not in Hardy-Weinberg equilibrium (P ranging from 0.05 to less than 0.001) and this was expected as the 175 individuals do not belong to a panmictic population. Mean observed heterozygosity (H_o) was 0.78, ranging from 0.16 (*locus* Hi22f12) to 0.91 (CH01g12 and CH04c07) (**Table 3**). Mean expected heterozygosity (H_e) was

TABLE 3 | Genetic diversity in terms of range of allele size (bp), number of allele (Na), effective number of alleles (Ne), percentage of rarity (RA), observed (Ho) and expected (He) heterozygosity, inbreeding coefficient (F), polymorphic information content (PIC) and probability of identity (P_{ID} and P_{IDsib}) of all 175 accessions of apple germplasm evaluated.

Locus	Range of allele size (bp)	Na	Ne	RA†	Ho	He	*F*	PIC	P_{ID}‡ unrelated	P_{IDsib}‡
CH05c06	104–134	14	4.68	28.6	0.80	0.79	−0.018	0.784	0.0183	0.3626
CH-Vf1	127–173	14	2.85	57.1	0.75	0.65	−0.154	0.647	0.0761	0.4453
Hi07h02	238–276	17	8.84	23.5	0.86	0.89	0.034	0.885	0.0025	0.3084
CH03d12	96–157	25	7.36	40.0	0.83	0.86	0.041	0.862	0.0091	0.3214
CH05e03	145–224	26	7.79	42.3	0.70	0.87	0.200	0.869	0.0059	0.3169
Hi04e04	208–249	15	6.75	20.0	0.87	0.85	−0.020	0.849	0.0088	0.3275
CH02b03b	73–106	13	6.43	15.4	0.90	0.84	−0.062	0.842	0.0074	0.3308
CH01f03b	137–185	11	4.83	18.2	0.88	0.79	−0.110	0.791	0.0163	0.3587
CH02c09	234–259	12	5.51	16.7	0.82	0.82	−0.005	0.816	0.0139	0.3453
Hi22f12	202–218	5	3.91	–	0.16	0.74	0.784	0.742	0.0307	0.3867
CH01g12	102–186	21	7.77	33.3	0.91	0.87	−0.043	0.869	0.0052	0.3169
AU223657	224–237	6	4.38	–	0.72	0.77	0.067	0.769	0.0180	0.3698
CH01h02	236–255	9	3.36	22.2	0.77	0.70	−0.098	0.701	0.0595	0.4146
CH01h01	104–131	12	6.70	25.0	0.85	0.85	0.000	0.849	0.0050	0.3270
CH04a12	159–202	18	4.53	27.8	0.81	0.78	−0.041	0.777	0.0322	0.3696
Hi03a10	199–291	16	8.89	18.8	0.75	0.89	0.156	0.885	0.0023	0.3080
CH04c07	95–139	16	8.06	12.5	0.91	0.88	−0.038	0.874	0.0039	0.3142
CH01c06	149–191	14	4.19	28.6	0.81	0.76	−0.065	0.759	0.0369	0.3796
CH02g01	184–246	14	6.09	14.3	0.80	0.84	0.036	0.834	0.0113	0.3360
Mean	–	**14.6**	**5.94**	–	**0.78**	**0.81**	**0.036**	**0.811**	–	–
Total	–	278	112.92	–	–	–	–	–	$\mathbf{2.2 \times 10^{-38}}$	$\mathbf{8.5 \times 10^{-10}}$

†*RA: percentage of rare alleles; an allele is defined rare at a frequency < 0.01;* ‡*The total value of the column is the cumulative $P_{(ID)}$ obtained as the product of the $P_{(ID)}$ of individual loci. The same is for $P_{(ID)sib}$.*

0.81, denoting high variability and ranging from 0.65 (CH-Vf1) to 0.89 (Hi07h02 and Hi03a10). F coefficients ranged from − 0.154 (CH-Vf1) to + 0.784 (Hi22f12), but the latter was the only value significantly departing from the others, thus denoting high homozygosity.

The 19 *loci* showed PIC values ranging from 0.65 (CH-Vf1) to 0.89 (Hi07h02 and Hi03a10), which were all higher than 0.5 and therefore very informative (Botstein et al., 1980).

The probability of identity (P_{ID}) of a *locus* is the probability that two individuals share the same genotype at that *locus*, while the power of discrimination (PD = 1−P_{ID}) is the probability that two individuals have different genotypes at that *locus*. An overall mean value of PD = 0.98 (ranging from 0.924 to 0.997) indicates that the *loci* are polymorphic enough in discriminating individuals. By considering the profile of the 19 *loci* at the same time, the probability to find two identical individuals is indeed remote ($P_{ID} = 2.2 \times 10^{-38}$); therefore, two individuals with the same profile at 19 *loci* are expected to be clones of the same genotype.

Genetic Structure and Identification of Unknown Accessions

The Ward's clustering method, based on the Euclidean distance matrix, highlighted several individuals grouped at zero distance and, according also to the P_{ID} value based on the polymorphisms at 19 *loci*, they can be considered to be the same genotype (**Supplementary Figure S2**). **Table 4** lists 38 accessions that were found to be identical. Among these, accessions #049, #012, #058, #147, and #013 were identical to #087, #010, #059, #118, and #043, respectively; they confirmed the correctness of the procedure as they had the same names, even if some of them were provided by different collections. Moreover, five unknown accessions, namely #076, #173, #148, #151, and #093 were identical to known genotypes and could therefore be named Annurca, Fuji, Coccianese, Rosciola, and Reinette du Canada, respectively. Some other accessions found to be identical can be considered synonyms: #002 of 001_Cerina, #134 of 126_Limoncella, #015 of 011_Sona, #028 and #030 of 016_Gelata, #041 of 039_Ciocarina Bianca, #053 of 080_ Ducale, #112 of 146_Gelata, #035, and #036 of 034_Panaia. Lastly, there were 5 other pairs of identical accessions (#055 and #060; #095 and #160; #103 and #157; #099 and #140; #054 and #102), all of unknown origin. In order to proceed with the statistical analysis accessions #054, #060, #103, #140, and #160 were removed.

After the removal of 25 duplicates, the 150 accessions were reanalyzed by Ward's clustering and analyzed by STRUCTURE. The results are reported in **Figure 1** and are aligned in order to compare and, eventually, validate accession membership and population structure. On Ward's dendrogram and at a distance of 60 units we found two main groups ($P < 0.001$). The upper group includes all local varieties, while the other includes several commercial varieties that were used in this study as controls: Annurca, Reinettes, Abbondanza, Golden Delicious, Golden Gala, Fuji, Cripps Pink, and Stark Delicious. Moreover, at a distance of 35 the dendrogram showed essentially

TABLE 4 | Accessions with identical SSR profile at 19 *loci*, used to identify unknown accessions and synonyms*.

Synonyms/Unknown	Reference accession	Cluster
049_Amerina	087_Amerina	1
012_Ruzza	010_Ruzza	2
058_Coccianese	059_Coccianese	3
147_Rosa in Pietra	118_Rosa in Pietra	3
013_San Giovanni	043_San Giovanni	5
148_Unknown	149_Coccianese	3
076_Unknown	136_Annurca	4
173_Unknown	171_Fuji	5
151_Unknown	082_Rosciola	5
093_Unknown	*131_Reinette du Canada*	5
112_Paradisa	146_Gelata	1
002_Zuccherina	001_Cerina	1
028_Cerina 030_Gelata	016_Gelata	1
015_A Sonagli	011_Sona	3
041_Ciochera Rosa	039_Ciocarina Bianca	3
134_Limoncina	126_Limoncella	3
053_Rosa d'Amelia	080_Ducale	3
035_Pagliaccia *036_Casciola*	*034_Panaia*	4

**Please note that other five pair of Unknown accessions were identical and only one was maintained for further analysis (see text in Results). Accessions in italics are putative polyploids.*

5 clusters, in agreement with $K = 5$ of STRUCTURE (see later paragraph).

Cluster 1 is characterized by a group of local varieties which refer to 'Gelata' (#146, #016, #029), common in central Italy where it is known with different names: Cerina, Oleata, Cera (#001, #027, #017, and #152, respectively) (Gallesio, 1817/1839; Molon, 1901; Tamaro, 1929). In this cluster there are also two very similar accessions (#104 and #107) from Montelupone (Marche), differing from one another only for the skin color, red, and yellow, respectively.

Cluster 2 includes several ancient accessions from Umbria, such as Ruzza (#066 and #010) or Roggia (#128 and #018), which had already been described in the 16th century (Gallo, 1540). In this cluster there are two accessions named 'Conventina' (#064 and #127) from Gubbio (Umbria), which is the Italian name for 'Monastery,' and where this variety was grown since the Middle Ages, and it is known to be suitable for mountain areas (Tonini, 1930). Lastly, there is a subcluster of Piattuccia (#062) and Pianella (#050, #081) whose names are due to a flattened fruit shape.

Cluster 3, with 45 accessions, includes the subgroups of Spoletina (#067, #139), of Limoncella (#126, #061, and #141), of several polyploids (red labeled beside the name), the local varieties Sona (#011), Coccianese (#059) and several unknown accessions. Limoncella is an ancient variety common in the South of Italy whose name means 'small lemon'; Sona is common in Valnerina (Umbria) and its name derives from the rattling sound of the seeds inside the carpel detaching at maturity.

Although apparently uniform, in Cluster 4 it is possible to detect two subgroups, one including all diploids accessions that refer to Annurca, an ancient cultivar of Campania whose origins were already described by Pliny the Elder (Pasquale, 1876), and the other including all polyploids and refers to Panaia (#034) and Polsola (#073), a synonym of Panaia, a local variety from Tuscany that spread in Umbria around 1700 and later in Abruzzo (Gallesio, 1817/1839). In the Annurca subgroup there are two unknown accessions, #096 and #111, tightly joined together ($P < 0.06$), both collected in Umbria.

Cluster 5 is the most numerous. It includes 54 accessions and it is possible to distinguish the group of Reinettes, the group of Golden, that of Appiola, that of Abbondanza and that of Fuji, Cripps Pink, and Stark Delicious. The Reinette accessions (#131, #132, and #133) and those known as 'Mele Grigie' ('gray apples,' from #019 to #024), provided by the Department of Agriculture of Torino, are very common in the North-West of Italy and are characterized by an acidic pulp and by a shrunken skin at maturity.

The plot of the average log-likelihood values for *K*s ranging from 1 to 21 and the distribution of ΔK-values (Evanno et al., 2005) according to *K*-values are shown in the **Supplementary Figure S3**. Two peaks were found, the first corresponding to $K = 2$ and the second to $K = 5$; the hierarchical genetic structure was investigated at $K = 5$. A threshold value of $P_{qI} \geq 0.80$ was used to assign individuals to the groups (**Figure 1**). With this threshold as many as 58 of 150 individuals were classified as admixture. At $K = 5$ STRUCTURE was able to define 5 groups, identified by the colors green, yellow, blue, red, and gray, respectively, which are represented side by side with those detected by Ward's clustering (**Figure 1**). By comparing the two grouping methods, and apart from the admixtures, it can be stated that there was a similar trend and correspondence. In Group 1 (green) only 7 out of 20 accessions were not classified as admixtures, and they all belong to Gelata and Cera (these are normally considered synonyms). In Group 2 (yellow) only 7 out of 18 accessions were classified at $P_{(qI)} \geq 0.80$; of these, some belong to the group of Ruzza (synonym of Roggia, #128, #066, #010, #018, Dalla Ragione and Dalla Ragione, 2011) and the rest to Piattuccia (#062, #110, #138), confirming their cluster closeness. In Group 3 (blue), the cluster of Spoletina, Sona and Limoncella, 28 out of 45 were correctly classified ($P_{(qI)} \geq 0.80$). In Group 4 (red) 11 out of 13 accessions were correctly classified: the Annurca's (all diploids) and a set of triploids, all belonging to #034_Panaia. Group 5 (gray) includes several commercial varieties (Reinettes, Golden Delicious, Golden Gala, Abbondanza, Fuji, Cripps Pink, and Stark Delicious) and here as many as 35 out of 54 accessions were accurately classified. Overall, by comparing the two methods, only two misclassified accessions emerged: the unknown #157 found in Ward's Cluster 3 but ascribed by STRUCTURE in Group 2 (yellow), and 039_Ciocarina Bianca in Cluster 5 attributed by STRUCTURE in Group 3 (blue).

Having established that 92 accessions showed a genetic structure, the five groups were compared in terms of number of alleles, expected and observed heterozygosity (**Table 5**) and F-Statistics (**Table 6**). However, the mean number of alleles

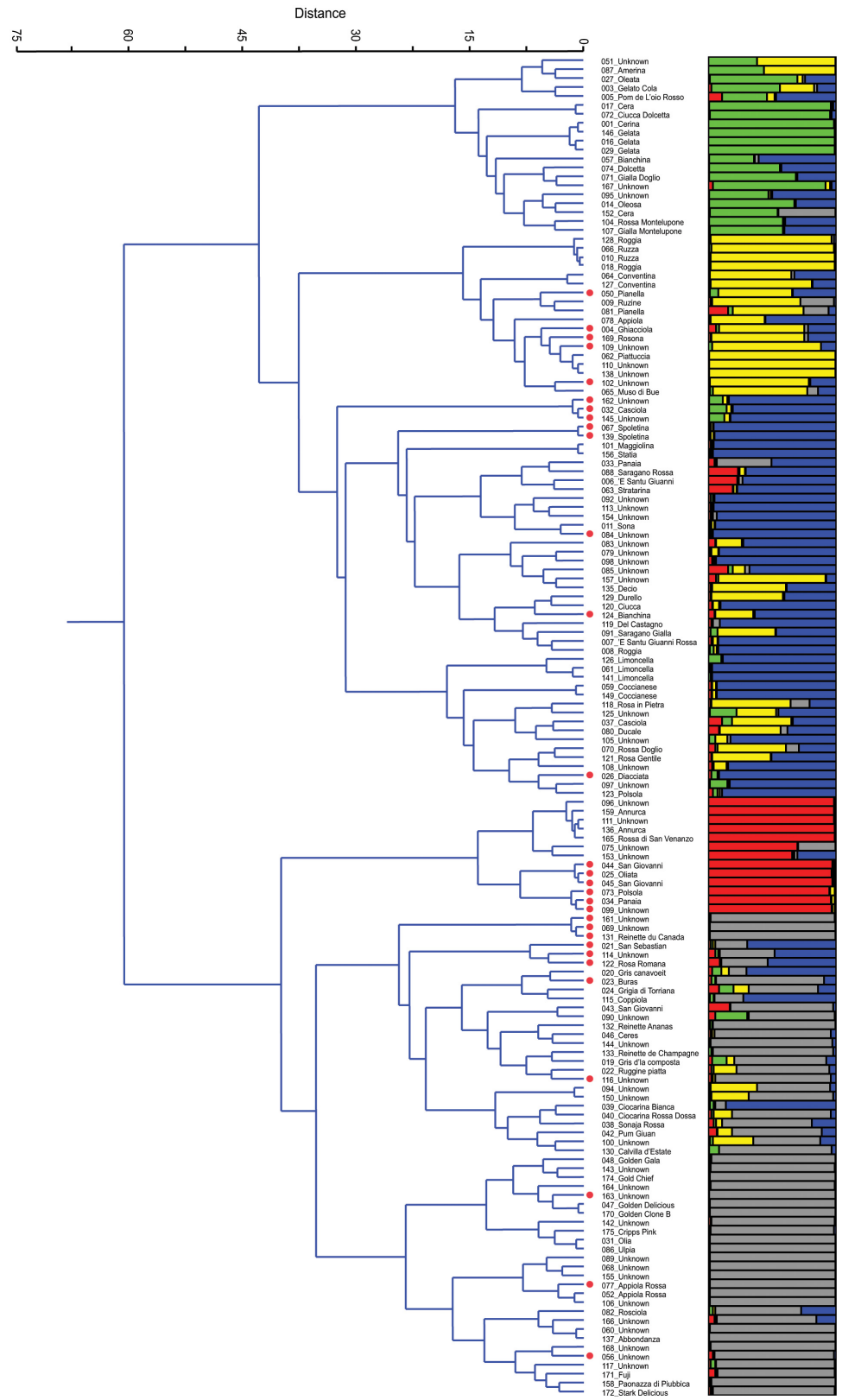

FIGURE 1 | Ward's clustering dendrogram of the 150 accessions of apple (on the left) and assignment by STRUCTURE at $K = 5$ (on the right). Polyploids are red labelled beside the accession name.

TABLE 5 | Number of alleles (Na), observed (H_o), and expected (H_e) eterozygosity for the five groups revealed by STRUCTURE.

	ALL (*n* = 92)			Group1 (*n* = 7)			Group2 (*n* = 9)			Group3 (*n* = 29)			Group4 (*n* = 11)			Group5 (*n* = 36)		
Locus	**Na**	**H_e**	**H_o**	**Na**	**H_e**	**H_o**	**Na**	**H_e**	**H_o**	**Na**	**H_e**	**H_o**	**Na**	**H_e**	**H_o**	**Na**	**H_e**	**H_o**
CH05c06	13	0.81	0.83	3	0.60	1.00	1[a]	0.00	0.00	10	0.77	0.90	6	0.73	1.00	9	0.79	0.89
CH-Vf1	10	0.64	0.74	2	0.49	0.71	2	0.11	0.11	7	0.71	0.90	3	0.65	1.00	7	0.63	0.69
Hi07h02	15	0.89	0.87	3	0.38	0.43	3	0.66	1.00	14	0.92	0.86	5	0.77	1.00	11	0.82	0.89
CH03d12	22	0.87	0.78	2	0.14	0.14	5	0.71	1.00	16	0.89	0.93	7	0.80	0.75	10	0.74	0.75
CH05e03	22	0.90	0.70	2	0.54	1.00	6	0.64	0.67	14	0.89	0.66	2	0.42	0.00	13	0.90	0.89
Hi04e04	14	0.88	0.85	2	0.44	0.57	3	0.45	0.56	9	0.83	0.97	5	0.70	1.00	9	0.86	0.83
CH02b03b	13	0.86	0.90	2	0.53	0.86	4	0.69	1.00	8	0.76	0.86	6	0.78	1.00	10	0.83	0.89
CH01f03b	11	0.79	0.89	4	0.71	1.00	4	0.69	1.00	8	0.81	0.90	3	0.50	0.55	8	0.81	0.94
CH02c09	12	0.85	0.85	4	0.67	1.00	1[b]	0.00	0.00	8	0.81	0.86	6	0.83	1.00	8	0.82	0.97
Hi22f12	5	0.76	0.13	3	0.54	0.14	3	0.57	0.11	5	0.75	0.18	1[c]	0.00	0.00	4	0.73	0.14
CH01g12	17	0.88	0.91	3	0.67	1.00	5	0.60	0.78	12	0.84	0.90	5	0.70	1.00	11	0.84	0.92
AU223657	6	0.78	0.67	2	0.26	0.29	3	0.66	1.00	5	0.78	0.79	3	0.63	1.00	4	0.47	0.47
CH01h02	9	0.71	0.75	3	0.66	0.86	5	0.71	1.00	8	0.74	0.72	3	0.45	0.55	5	0.72	0.75
CH01h01	11	0.85	0.88	2	0.36	0.43	4	0.71	1.00	11	0.87	0.90	5	0.78	1.00	7	0.81	0.89
CH04a12	14	0.80	0.80	3	0.66	0.86	3	0.57	0.89	10	0.81	0.76	4	0.46	0.55	8	0.80	0.89
Hi03a10	15	0.88	0.75	3	0.63	0.71	3	0.69	1.00	13	0.90	0.66	4	0.61	0.73	9	0.76	0.78
CH04c07	16	0.89	0.92	3	0.69	1.00	4	0.71	1.00	12	0.83	0.90	7	0.87	1.00	9	0.85	0.89
CH01c06	11	0.76	0.83	1[d]	0.00	0.00	3	0.66	1.00	9	0.75	0.93	3	0.50	0.55	8	0.83	0.94
CH02g01	13	0.82	0.77	3	0.69	1.00	3	0.63	0.89	9	0.80	0.86	4	0.71	0.55	8	0.74	0.69
Mean	13.1	0.82	0.78	2.6	0.51	0.68	3.4	0.55	0.74	9.9	0.81	0.81	4.3	0.63	0.75	8.3	0.78	0.80

[a]*locus with a fixed allele 122;* [b]*locus with a fixed allele 248;* [c]*locus with a fixed allele 218;* [d]*locus with a fixed allele 163.*

across the 19 *loci* and for all 92 accessions, as revealed by STRUCTURE, was 13.1, very similar to the population based on 150 accessions (14.6); likewise were the values of H_e (0.82 for both) and H_o (0.79 *vs*. 0.78, respectively). As expected, the mean number of alleles per *locus* in Groups 1, 2, and 4 was consistently lower than those for Groups 3 and 5, since heavily dependent on the number of accession forming the groups. Interestingly, in the former, less numerous groups, there was at least one *locus* with a fixed allele (**Table 5**), hence the indexes of diversity (H_e) were lower than those of Group 3 and 5. As expected, the overall F_{IS} of the 5 groups was slightly negative (outbreeding), consistent for the majority of the *loci* (14), except for CH05e03 (0.1045, $P < 0.05$) and especially for Hi22f12 (0.7914, $P < 0.001$), highly homozygous compared with the expected values. The overall *loci* F_{ST}-value (0.1470, $P < 0.001$) is to be considered rather high, meaning that the 92 accessions were well structured and close to a value (0.15), generally considered to indicate a threshold limit between a moderate and a great differentiation (Wright, 1978). However, upon closer inspection of the F_{ST}-values at each *locus* it is possible to note that, apart from those *loci* whose values are close to the mean, some exceeded it by at least 3 times the standard error; these were CH05c06, Hi22f12, and AU223657 with 0.1995, 0.2162, and 0.3074, respectively, all significant at $P < 0.001$. Some other *loci* showed values more than 3 times lower than the mean; they were CH04c07, CH01h01, CH-Vf1, and CH01f03b, all significant at $P < 0.001$. In particular *locus* CH01h02 with a F_{ST}-value of 0.0403 ($P < 0.05$) denoted a little differentiation. All the results reported above were confirmed by AMOVA, where the variation within groups was 75.9% and among was 24.1%, values different from those reported in literature (Gasi et al., 2010; Urrestarazu et al., 2012, 2016; Pereira-Lorenzo et al., 2017).

Parentage Analysis

The parentage analysis was used (i) to investigate the origins of the 49 unknown accessions after the removal of 10 found identicals to well-known genotypes and (ii) to look for concordance with the results from STRUCTURE and Ward's clustering.

Table 7 reports the 38 unknown accessions significantly related (LOD score > 5) to parents of known origin (LV and CV). As many as 27 of them showed full concordance with STRUCTURE and Ward's clustering. The most likely parents of 10 unknown accessions, classified by STRUCTURE into the Admixture group, were classified also by Ward's in the same group. Lastly, the 144_Unknown was included by STRUCTURE in group 5, whereas the most likely parent, 033_Panaia, was classified differently by the other two analytic procedures (Ward's Cluster3 and Admixture in STRUCTURE).

In particular, 051_Unknown was classified by STRUCTURE as admixture, showing a probability of 0.38 to be assigned to Group1 and of 0.61 to Group2; likely parents were 001_Cerina and 062_Piattuccia, belonging to Cluster1/Group1 and to Cluster2/Group2, respectively. Since Ward's clustering assigned #051 to Cluster 1, it can be stated that STRUCTURE was more efficient to infer its mixed genomic configuration.

TABLE 6 | F statistics and significance for each *locus* and total of the 5 groups as obtained by STRUCTURE (SPAGeDi).

Locus	F_{IS}‡	F_{ST}
CH05c06	−0.2264 ***	0.1995***
CH-Vf1	−0.2398 ***	0.0894***
Hi07h02	−0.1029 *	0.1346***
CH03d12	−0.0687 n.s.	0.1659 ***
CH05e03	0.1045 *	0.1546 ***
Hi04e04	−0.1200 ***	0.1635 ***
CH02b03b	−0.1988 ***	0.1423 ***
CH01f03b	−0.1968 ***	0.0670 ***
CH02c09	−0.1791 ***	0.1849 ***
Hi22f12	0.7914 ***	0.2162 ***
CH01g12	−0.1736 ***	0.1403 ***
AU223657	−0.1676 ***	0.3074 ***
CH01h02	−0.1151 *	0.0403 *
CH01h01	−0.1458 ***	0.1050 ***
CH04a12	−0.1146 *	0.1166 ***
Hi03a10	0.0006 n.s.	0.1547 ***
CH04c07	−0.1363 **	0.1084 ***
CH01c06	−0.2209 ***	0.1360 ***
CH02g01	−0.0646 n.s.	0.1324 ***
Mean	−0.0866 ***	0.1470***
SE	0.0460	0.0127

‡*F_{IS} is the loss of heterozygosity due to inbreeding, F_{ST} is the loss of heterozygosity due to genetic drift; *, **, ***F-values significant at $P < 0.05$, 0.01, and 0.001, respectively; n.s. not significant.*

Correlation of SSR Alleles and Some Morphological Traits

Correlations among morphological traits were not significant, except over color *vs.* fruit rustiness ($r = -0.3166$, $P < 0.001$). Eleven out of 19 SSR *loci* revealed that 15 alleles out of 278 were significantly ($P < 0.001$) correlated with five morphological traits (**Table 8**). Four alleles showed a significant negative correlation with time of eating maturity, so that the presence of these alleles was related with early maturity. CH03d12_128 and Hi03a10_199 were positively correlated with fruit shape. Similarly, pulp color revealed two alleles with significant positive correlations with two SSR *loci* related to aroma compounds (Dunemann et al., 2009), indicating putative correlation between pulp color and aroma trait.

The over color was found negatively correlated with several alleles, with *r*-values ranging from − 0.3049 (for CH02b03b_077) to − 0.36308 (for CH_Vf1_127), meaning that their presence is by some means related to light colors (absence, yellow, and orange). Interestingly, the majority of the marker-alleles detected for the over color were related to QTLs for resistance to biotic stresses, aroma compounds, stiffness, and acidity, indicating a possible correlation among these traits (Kenis et al., 2008). Lastly, only one allele (CH01f03b_137) resulted positively correlated with ground color.

Moreover, three (CH01g12, Hi03a10, and Hi04e04) out of eleven *loci* identified in the correlation test were found in at least two traits, while CH01g12 was detected in maturity, over color and pulp color.

DISCUSSION

The rapid spread of modern, intensive agricultural techniques during the last century was also accompanied by a rapid spread of newly bred cultivars characterized by greater productivity and uniformity. Although this trend was more intensive in annual crops, it did not spare fruit orchards. The oversimplification of the agricultural systems in favorable areas caused parallel changes in the agricultural economy, farming assets, rural culture, and agricultural landscapes as well. From a biological point of view this determined a significant reduction of crop biodiversity and a progressive genetic erosion with the loss of many ancient, well adapted local varieties. For this reason, over the last 50 years, the need to develop effective strategies for the conservation and management of genetic resources has become a fundamental issue. At this purpose germplasm banks have been established all over the world, operating at international, national and local levels. Italy, in compliance with EU directives, has promoted several Regional germplasm collections, with the aim of maintaining and preserving the autochthonous diversity. In Italy fruit germplasm, and apple genetic resources in particular, are conserved by National and Regional institutions (such as CREA, University of Bologna, Malva Rinaldi School of Torino, and many others), several of whom kindly provided accessions that were used as controls in this study.

The area of Central Italy is characterized by hills, mountains, small valleys, a variety of soil types (Corti et al., 2013) and of exposure, generating many micro-environments. Rainfall amounts and distribution from the East to the West coasts, passing through the Apennines, is also different, as well as the temperatures due to altitude differences from sea level to 2000–3000 m a.s.l. (Longinelli and Selmo, 2003). Fruit in general, and apples in particular, were rarely grown in specialized and intensive cultivation over large areas. Therefore, coupling these conditions together can perhaps explain most of the reasons at the base of the rich diversity found in the apple germplasm of Central Italy, a picture difficult to find in other Italian regions.

Many of these local varieties are well adapted to specific agro-climatic conditions and often express some diversity with respect to the originals in terms of morphological and physiological traits, thus assuming different names. Often, the names were assigned on the base of phenotypic traits, strongly influenced by the environment and agricultural practice, thus increasing the existing confusion about local genetic resources and their correct denomination. This gives rise to the importance of characterizing the germplasm present in the national and regional germplasm banks, by identifying duplicates and redundant accessions, hence simplifying the management and reducing costs of living collections.

For this purpose, molecular markers and in particular SSR have been widely used in genetic diversity studies and clarified cases of synonymy and homonymy in core collections (Patzak et al., 2012; Urrestarazu et al., 2012, 2016; Liang et al., 2015; Yun et al., 2015; Lassois et al., 2016). Following the detection of null alleles, two of the initial 21 *loci* (Hi23g02 and Hi03g02) were discarded. The remaining 19 showed a high degree of

TABLE 7 | Parentage analysis of unknown accessions, their membership to the group of Stucture, the most likely parent of known origin (LOD score as otained by FaMoz) and its membership to Cluster and to STRUCTURE.

Unknown accessions	Classified by STRUCTURE into Group	Likely parents of known origin	LOD score	Ward's Cluster Number	Classified by STRUCTURE into Group
#051	Adm	#001 Cerina	12.79	1	1
		#062 Piattuccia	11.56	2	2
#167	1	#016 Gelata	15.34	1	1
		#029 Gelata	14.65	1	1
		#146 Gelata	11.69	1	1
		#001 Cerina	11.00	1	1
#095	Adm	#001 Cerina	9.27	1	1
		#146 Gelata	5.00	1	1
#109	2	#062 Piattuccia	17.87	2	2
#110	2	#062 Piattuccia	22.50	2	2
		#004 Ghiacciola	16.29	2	2
#138	2	#062 Piattuccia	23.33	2	2
		#004 Ghiacciola	17.00	2	2
#102	Adm	#062 Piattuccia	17.91	2	2
#162	3	#032 Casciola	21.81	3	3
#145	3	#032 Casciola	23.40	3	3
#084	3	#011 Sona	22.84	3	3
#079	3	#135 Decio	14.69	3	3
#097	3	#123 Polsola	10.44	3	3
#096	4	#165 Rossa di San Venanzo	24.47	4	4
		#159 Annurca	23.52	4	4
		#136 Annurca	23.41	4	4
#111	4	#159 Annurca	24.27	4	4
		#136 Annurca	24.16	4	4
#075	Adm	#136 Annurca	16.78	4	4
#153	Adm	#159 Annurca	11.17	4	4
		#136 Annurca	11.05	4	4
#099	4	#073 Polsola	30.01	4	4
		#034 Panaia	27.96	4	4
#161	5	#131 Reinette du Canada	25.20	5	5
		#019 Gris d'la Composta	6.04	5	5
#069	5	#131 Reinette du Canada	25.88	5	5
		#019 Gris d'la Composta	6.72	5	5
#090	Adm	#043 San Giovanni PT	21.60	5	5
#144	5	#033 Panaia	8.72	3	Adm
#116	5	#131 Reinette du Canada	6.37	5	5
#094	Adm	#038 Sonaja Rossa	7.04	5	5
#150	Adm	#038 Sonaja Rossa	5.93	5	5
#100	Adm	#130 Calvilla d'Estate	14.04	5	5
#143	5	#047 Golden Delicious	19.31	5	5
		#170 Golden Clone B	18.74	5	5
		#174 Gold Chief	13.66	5	5
		#172 Stark Delicious	12.47	5	5
		#171 Fuji	7.99	5	5
		#048 Golden Gala	6.47	5	5
#164	5	#047 Golden Delicious	11.50	5	5
		#170 Golden Clone B	10.81	5	5
		#175 Cripps Pink	9.13	5	5
		#174 Gold Chief	5.58	5	5

(Continued)

TABLE 7 | Continued

Unknown accessions	Classified by STRUCTURE into Group	Likely parents of known origin	LOD score	Ward's Cluster Number	Classified by STRUCTURE into Group
#163	5	#170 Golden Clone B	16.89	5	5
		#047 Golden Delicious	13.56	5	5
		#174 Gold Chief	12.67	5	5
		#137 Abbondanza	6.82	5	5
#142	5	#086 Ulpia	15.32	5	5
		#031 Olia	13.71	5	5
#089	5	#052 AppiolaRossa	16.14	5	5
		#137 Abbondanza	13.24	5	5
		#077 Appiola Rossa	7.25	5	5
#068	5	#137 Abbondanza	19.26	5	5
		#052 AppiolaRossa	13.67	5	5
#155	5	#137 Abbondanza	16.43	5	5
		#052 AppiolaRossa	11.63	5	5
		#158 Paonazza di Piubbica	6.92	5	5
#106	5	#052 AppiolaRossa	30.40	5	5
		#077 Appiola Rossa	22.15	5	5
#166	Adm	#137 Abbondanza	19.49	5	5
#060	5	#137 Abbondanza	33.23	5	5
		#158 Paonazza di Piubbica	13.17	5	5
#168	5	#052 AppiolaRossa	15.94	5	5
		#172 Stark Delicious	15.77	5	5
		#077 Appiola Rossa	6.25	5	5
		#158 Paonazza di Piubbica	5.80	5	5
#056	5	#077 Appiola Rossa	8.41	5	5
		#172 Stark Delicious	8.00	5	5
		#052 AppiolaRossa	7.95	5	5
#117	5	#172 Stark Delicious	17.69	5	5
		#174 Gold Chief	6.22	5	5

TABLE 8 | Spearman correlation coefficients (*r*) and statistical significance (*P*-value) between SSRs *loci* and morphological traits.

Traits	SSR locus_allele	*r*	*P*-value
Time of eating maturity	CH05c06_106	−0.28146	0.0010
	CH02c09_234	−0.28809	0.0008
	CH01g12_146	−0.28636	0.0008
	AU223657_235	−0.28460	0.0009
Fruit shape	CH03d12_128	0.34733	0.0001
	Hi03a10_199	0.29298	0.0006
Ground color	CH01f03b_137	0.28016	0.0010
Over color	CH_Vf1_127	−0.36308	0.0001
	Hi04e04_222	−0.31808	0.0002
	CH02b03b_077	−0.30490	0.0004
	CH01g12_151	−0.31013	0.0003
	Hi03a10_253	−0.31440	0.0002
	CH01c06_159	−0.31436	0.0002
Pulp color	Hi04e04_247	0.38470	0.0001
	CH01g12_146	0.33177	0.0001

polymorphism and discriminating power and allowed us to meet our objectives.

The pool of accession studied here showed a percentage of polyploids of 20%, a value intermediate between 8% reported by Urrestarazu et al. (2016) in screening a wide European collection, and those found in Spain: 34% by Pereira-Lorenzo et al. (2007), 29% by Ramos-Cabrer et al. (2007), and 24% by Urrestarazu et al. (2012).

In brief, our study showed that 25 accessions were duplicates, 9 had to be considered synonyms (**Table 4**) and 9 homonyms. Six accessions from the living collection of 3A-PTA Pantalla and 3A-PTA Casalina (Amerina c02_049/ c02_087, Coccianese c02_058/ c02_059, and Rosa in Pietra c02_147/c03_118) were duplicates of the same genotype, because the SSR profiles were identical throughout the 19 *loci*. Also San Giovanni (c02_013) and Ruzza (c02_012) turned out to be identical to the corresponding accessions provided by the Department of Agricultural Science, University of Bologna (c08) and the National Center of Fruit Tree Germplasm (c01), thus confirming the goodness of the analysis.

Among polyploids we found some accessions named Panaia or Polsola, and they are synonyms (Dalla Ragione and Dalla Ragione, 2011). This ancient variety, whose local name 'Panaia' derives from 'bread basket,' was very common in Central

Italy and in the past two varieties were described by Gallesio (1817/1839): 'Panaia massima' and 'Panaia a frutto piccolo.' These denominations refer to the dimension of the fruit, and may explain the homonymy between #034 and #075 (polyploids with bigger fruit) vs. #033 (diploid with smaller fruit).

As far as homonymy is concerned, three accessions named 'San Giovanni' were also scattered among Clusters 4 and 5. This is a group of accessions whose local name is linked to the time of maturation (end of June), independently of other traits, one is diploid (#043, Cluster 5) while the other two were polyploids (#044, #045, Cluster 4). Other examples of homonymy include #008_Roggia (Cluster3), genetically different from the two Roggia listed in Cluster2; Appiola Rossa #052 (diploid) vs. Appiola Rossa #077 (polyploid) in Cluster 5, and Bianchina #057 (diploid, in Cluster1) vs. Bianchina #124 (polyploid, in Cluster 3).

Another significant result of our study was the genetic identification of several unknown accessions. Ten of them were excluded as duplicates of well-known accessions. By using different statistical approaches (Cluster, STRUCTURE and Parentage analysis) it was possible to assign 37 more accessions to known commercial or local varieties.

Lastly, it is worth mentioning that the 92 accessions found by the Bayesian analysis were well-structured at $K = 5$, where the F_{ST}-value indicates a high differentiation among subpopulations, much higher than those reported in the literature (Pereira-Lorenzo et al., 2007; Gasi et al., 2010; Urrestarazu et al., 2012, 2016), indicating that the material from Central Italy is a genetic pool worthy of safeguarding and conservation. In particular we found that F_{ST} at some *loci* were very contrasting (see **Table 6**). The low F_{ST}-values at *loci* CH04c07, CH01h01, CH-Vf1, CH01f03b and CH01h02 suggests that homogenizing selection across subpopulations reduces differentiation, whereas the high F_{ST}-values at *loci* CH05c06, Hi22f12, and AU223657 suggest that selection for local adaptation is creating differentiation. In these cases we found that the allele 128 of CH-Vf1 is correlated with fruit over color and allele 137 of *locus* CH01f03b with fruit ground color, while allele 106 of CH05c06 and allele 235 of AU223657 are correlated with time of eating maturity (**Table 8**).

Unexpectedly, the observed heterozygosity at the *locus* Hi22f12 was significantly lower than the values at the other 18 *loci* (0.13 *vs.* 0.78), meaning that almost all individuals at this *locus* are homozygous. Actually, this is also the *locus* with the lowest number of alleles. The sequence of the Hi22f12 SSR *locus* was then used in the BLAST program analysis against the NCBI nr database, and we found that Hi22f12 is located inside the transcription factor IIE subunit 1-like gene (XM_008376494.2) of *Malus* × *domestica*, at the position 1187–1242. The expectation and the identity of the query against the reference gene was 2×10^{-6} and 85%, respectively. The stability needed by this gene explains this low polymorphism, perhaps confined to introns. It would be interesting to extend this investigation to other germplasm collections.

In the present study Hi22f12 and some other SSR *loci* resulted fixed; in particular, *loci* CH05C06 and CH02c09 showed a fixed allele in group 2 of STRUCTURE. Of these, CH05C06 is of particular interest, associated with a major QTL for fruit titratable acidity (TA) detected in the *Ma* region (Liebhard et al., 2003; Kenis et al., 2008). In the cross 'Telamon × Braeburn,' this QTL was mapped in the LG16, an interval between the markers CH05e04z and CH05c06 and explained 20–34% of the observed variance (Kenis et al., 2008). The *Ma* gene controls the level of malic acid in apples and many other fruits (Maliepaard et al., 1998; Liebhard et al., 2003; Xu et al., 2012). Indeed, acidity is one of the most important fruit traits and, in apples, it strongly affects quality and organoleptic characteristics. In fact, the balance between sugars and acids is the basis of the taste and flavor of fruit (Wu et al., 2007; Zhang et al., 2010) and is therefore of utmost importance in breeding programs (Visser and Verhaegh, 1978). Moreover, this *locus* seems to be associated with a second QTL (M2), coding for the aromatic compound β-damascenone (Dunemann et al., 2009), a potent aroma present in apples (Cunningham et al., 1986; Fuhrmann and Grosch, 2002) and other fruits (peaches and grapes) and beverages (coffee, beer, and wine), and is associated with descriptions such as "fruity-flowery," and in particular "apple" and "baked apple" (Pineau et al., 2007). In our study the allele 120 at the *locus* CH05C06 is fixed in all accessions of Group 2 of STRUCTURE, the one hosting Conventina, Ruzza, Roggia, Rosona, and Piattuccia, all characterized by crispy, sugary, sour, and very aromatic pulp. In the same group 2 we found fixed also the allele 248 of the *locus* CH2c09 and this *locus* is linked to a QTL for the aromatic compound allylanil (M1) associated with anise and licorice descriptors on the LG 15(Plotto and McDaniel, 2000; Dunemann et al., 2009). Two other allele in the present investigation, the 162 at *locus* CH01c06 and the 218 at *locus* Hi22f12, were also fixed in Group 1 and Group 4, respectively, but no information was found in the *Malus* database.

CONCLUSION

In conclusion, this paper highlights the presence of considerable genetic variability among the apple accessions recovered in Central Italy and the information obtained can be used to better manage large living collections of a fruit tree of great nutritional interest such as the apple.

AUTHOR CONTRIBUTIONS

EA, LC, and FV conceived the study. EA and GM designed and coordinated the experiments. EA, GM, and LC chose and provided the germplasm. GM and NF performed the lab experiments. GM, LR, and NF conducted the data analysis and wrote the manuscript, while EA and FV critically reviewed it.

FUNDING

The research was supported by "*Convenzione con il Parco 3APTA 2014: Servizio di Conservazione e Ampliamento delle Banche Regionali della Biodiversità,*" CUP I61J11000280009.

ACKNOWLEDGMENTS

The authors would like to thank all the public and private institutions that kindly provided the accessions (listed in **Table 1**), Isabella Dalla Ragione and Mauro Gramaccia for technical assistance, and Liam Boyle for proofreading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01460/full#supplementary-material

REFERENCES

Albertini, E., Dalla Ragione, I., Falcinelli, M., Ferradini, N., Gramaccia, M., Lorenzetti, S., et al. (eds). (2015). "Il contributo delle analisi genetiche allo studio ed alla conoscenza della diversità delle varietà locali di melo," in *La Biodiversità di Interesse Agrario Della Regione Umbria - Specie Arboree da Frutto*, Vol. 2, (Todi PG: 3A-PTA), 12–57.

Assomela-CSO (2017). Available at: http://www.assomela.it/index_it.html

Blair, M. W., Díaz, L. M., Buendía, H. F., and Duque, M. C. (2009). Genetic diversity, seed size associations and population structure of a core collection of common beans (*Phaseolus vulgaris* L.). *Theor. Appl. Genet.* 119, 955–972. doi: 10.1007/s00122-009-1064-8

Botstein, D., White, R. L., Skolnik, M., and Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32, 314–331.

Breton, C., Pinatel, C., Médail, F., Bonhomme, F., and Bervillé, A. (2008). Comparison between classical and Bayesian methods to investigate the history of olive cultivars using SSR-polymorphisms. *Plant Sci.* 175, 524–532. doi: 10.1016/j.plantsci.2008.05.025

Caballero, A., and García-Dorado, A. (2013). Allelic diversity and its implications for the rate of adaptation. *Genetics* 195, 1373–1384. doi: 10.1534/genetics.113.158410/-/DC1

Celton, J. M., Tustin, D. S., Chagné, D., and Gardiner, S. E. (2009). Construction of a dense genetic linkage map for apple rootstocks using SSRs developed from Malus ESTs and Pyrus genomic sequences. *Tree Genet. Genomes* 5, 93–107. doi: 10.1007/s11295-008-0171-z

Cornille, A., Giraud, T., Smulders, M. J., Roldan-Ruiz, I., and Gladieux, P. (2014). The domestication and evolutionary ecology of apples. *Trends Genet.* 30, 57–65. doi: 10.1016/j.tig.2013.10.002

Cornille, A., Gladieux, P., Smulders, M. J., Roldan-Ruiz, I., Laurens, F., Le Cam, B., et al. (2012). New insight into the history of domesticated apple: secondary contribution of the European wild apple to the genome of cultivated varieties. *PLoS Genet.* 8:e1002703. doi: 10.1371/journal.pgen.1002703

Corti, G., Cocco, S., Brecciaroli, G., Agnelli, A., and Seddaiu, G. (2013). "Italian soil management from antiquity to nowadays," in *The Soils of Italy. World Soils Book Series*, eds E. A. C. Costantini and C. Dazzi (Dordrecht: Springer Netherlands), 247–293. doi: 10.1007/978-94-007-5642-7_9

Cunningham, D. G., Acree, T. E., Barnard, J., Butts, R. M., and Braell, P. A. (1986). Charm analysis of apple volatiles. *Food Chem.* 19, 137–147. doi: 10.1016/0308-8146(86)90107-X

Dalla Ragione, L., and Dalla Ragione, I. (2011). *Archeologia Arborea. Diario di due cercatori di piante*. Perugia: Alieno ed.

Dunemann, F., Ulrich, D., Boudichevskaia, A., Grafe, C., and Weber, W. E. (2009). QTL mapping of aroma compounds analysed by headspace solid-phase microextraction gas chromatography in the apple progeny 'Discovery' × 'Prima'. *Mol. Breed.* 23, 501–521. doi: 10.1007/s11032-008-9252-9

Earl, D. A., and vonHoldt, B. M. (2012). Structure Harvester: a website and program for visualizing structure output and implementing the Evanno method. *Conserv. Genet. Resour.* 4, 359–361. doi: 10.1007/s12686-011-9548-7

Emanuelli, F., Lorenzi, S., Grzeskowiak, L., Catalano, V., Stefanini, M., Troggio, M., et al. (2013). Genetic diversity and population structure assessed by SSR and SNP markers in a large germplasm collection of grape. *BMC Plant Biol.* 13:39. doi: 10.1186/1471-2229-13-39

Evanno, G., Regnaut, S., and Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Mol. Ecol.* 14, 2611–2620. doi: 10.1111/j.1365-294X.2005.02553.x

Evett, I. W., and Weir, B. S. (1998). *Interpreting DNA Evidence: Statistical Genetics for Forsenic Scientist*. Sunderland, MA: Sinauer Associates, Inc.

Excoffier, L., Smouse, P. E., and Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131, 479–491.

Falush, D., Stephens, M., and Pritchard, J. K. (2007). Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Mol. Ecol. Notes* 7, 574–578. doi: 10.1111/j.1471-8286.2007.01758.x

Food and Agricultural Organization of the United Nation [FAO] (2016). Available at: http://faostat.fao.org/beta/en/#data/QC/visualize

Fuhrmann, E., and Grosch, W. (2002). Character impact odorants of the apple cultivars Elstar and Cox Orange. *Mol. Nutr. Food Res.* 46, 187–193.

Gallesio, G. (1817/1839). *Pomona Italiana, Ossia Trattato Degli Alberi da Frutto*. Pisa: Nicolò Capurro.

Gallo, M. A. (1540). "Le vinti giornate dell'agricoltura et de' piaceri della villa," in *Anonymous Printing at Venice in the Sixteenth Century*, ed. D. E. Rhodes (Venetia: appresso Camillo, & Rutilio Borgomineri fratelli, al segno di San Giorgio).

Gao, Y., Liu, F., Wang, K., Wang, D., Gong, X., Liu, L., et al. (2015). Genetic diversity of *Malus* cultivars and wild relatives in the Chinese National Repository of Apple Germplasm Resources. *Tree Genet. Genomes* 11:106. doi: 10.1007/s11295-015-0913-7

Gasi, F., Simon, S., Pojskic, N., Kurtovic, M., and Pejic, I. (2010). Genetic assessment of apple germplasm in Bosnia and Herzegovina using microsatellite and morphologic markers. *Sci. Hortic.* 126, 164–171. doi: 10.1016/j.scienta.2010.07.002

Gerber, S., Chabrier, P., and Kremer, A. (2003). FaMoz: a software for parentage analysis using dominant, codominant and uniparentally inherited markers. *Mol. Ecol. Notes* 3, 479–481. doi: 10.1046/j.1471-8286.2003.00439.x

Gerber, S., Streiff, R., Bodénès, C., Mariette, S., and Kremer, A. (2000). Comparison of microsatellites and AFLP markers for parentage analysis. *Mol. Ecol.* 9, 1037–1048. doi: 10.1046/j.1365-294x.2000.00961.x

Gharghani, A., Zamani, Z., Talaie, A., Oraguzie, N. C., Fatahi, R., Hajnajari, H., et al. (2009). Genetic identity and relationships of Iranian apple (*Malus* x domestica Borkh) cultivars and landraces, wild *Malus* species and representative old apple cultivars based on simple sequence repeat (SSR) marker analysis. *Genet. Resour. Crop Evol.* 56, 829–842. doi: 10.1007/s10722-008-9404-0

Gianfranceschi, L., Seglias, N., Tarchini, R., Komjanc, M., and Gessler, C. (1998). Simple sequence repeats for the genetic analysis of apple. *Theor. Appl. Genet.* 96, 1069–1076. doi: 10.1007/s001220050841

Goulão, L., and Oliveira, C. M. (2001). Molecular characterisation of cultivars of apple (*Malus* × *domestica* Borkh.) using microsatellite (SSR and ISSR) markers. *Euphytica* 122, 81–89. doi: 10.1023/A:1012691814643

Gross, B. L., Henk, A. D., Richards, C. M., Fazio, G., and Volk, G. M. (2014). Genetic diversity in *Malus* × *domestica* (Rosaceae) through time in response to domestication. *Am. J. Bot.* 101, 1770–1779. doi: 10.3732/ajb.1400297

Gross, B. L., Volk, G. M., Richards, C. M., Forsline, C. L., Fazio, G., and Chao, C. T. (2012). Identification of "duplicate" accessions within the USDA-ARS national plant germplasm system *Malus* collection. *J. Am. Soc. Hortic. Sci.* 137, 333–342.

Guilford, P., Prakash, S., Zhu, J. M., Rikkerink, E., Gardiner, S., Bassett, H., et al. (1997). Microsatellites in *Malus domestica* (apple) abundance, polymorphism and cultivar identification. *Theor. Appl. Genet.* 94, 249–254. doi: 10.1007/s001220050407

Gygax, M., Gianfranceschi, L., Liebhard, R., Kellerhals, M., Gessler, C., and Patocchi, A. (2004). Molecular markers linked to the apple scab resistance gene Vbj derived from *Malus baccata* jackii. *Theor. Appl. Genet.* 109, 1702–1709. doi: 10.1007/s00122-004-1803-9

Hammer, K., Arrowsmith, N., and Gladis, T. (2003). Agrobiodiversity with emphasis on plant genetic resources. *Naturwissenschaften* 90, 241–250. doi: 10.1007/s00114-003-0433-4

Hammer, Ø, Harper, D. A. T., and Ryan, P. D. (2001). *Past: Paleontological Statistics Software Package for Education and Data Analysis*. Available at: http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Han, Y., and Korban, S. S. (2008). An overview of the apple genome through BAC end sequence analysis. *Plant Mol. Biol.* 67, 581–588. doi: 10.1007/s11103-008-9321-9

Hardy, O. J., and Vekemans, X. (2002). SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Mol. Ecol. Notes* 2, 618–620. doi: 10.1046/j.1471-8286.2002.00305.x

Harris, S. A., Robinson, J. P., and Juniper, B. E. (2002). Genetic clues to the origin of the apple. *Trends Genet.* 18, 426–430. doi: 10.1016/S0168-9525(02)02689-6

Holleley, C. E., and Geerts, P. G. (2009). Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *Biotechniques* 46, 511–517. doi: 10.2144/000113156

Kenis, K., Keulemans, J., and Davey, M. W. (2008). Identification and stability of QTLs for fruit quality traits in apple. *Tree Genet. Genomes* 4, 647–661. doi: 10.1007/s11295-008-0140-6

Kitahara, K., Matsumoto, S., Yamamoto, T., Soejima, J., Kimura, T., Komatsu, H., et al. (2005). Parent identification of eight apple cultivars by S-RNase analysis and simple sequence repeat markers. *HortScience* 40, 314–317.

Kloosterman, A. D., Budowle, B., and Daselaar, P. (1993). PCR-amplification and detection of the human D1S80 VNTR locus. *Int. J. Legal Med.* 105, 257–264. doi: 10.1007/BF01370382

Kunihisa, M., Moriya, S., Abe, K., Okada, K., Haji, T., Hayashi, T., et al. (2014). Identification of QTLs for fruit quality traits in Japanese apples: QTLs for early ripening are tightly related to preharvest fruit drop. *Breed. Sci.* 64, 240–251. doi: 10.1270/jsbbs.64.240

Lassois, L., Denancé, C., Ravon, E., Guyader, A., Guisnel, R., Hibrand-Saint-Oyant, L., et al. (2016). Genetic diversity, population structure, parentage analysis, and construction of core collections in the French apple germplasm based on SSR markers. *Plant Mol. Biol. Rep.* 34, 827–844. doi: 10.1007/s11105-015-0966-7

Liang, W., Dondini, L., De Franceschi, P., Paris, R., Sansavini, S., and Tartarini, S. (2015). Genetic diversity, population structure and construction of a core collection of apple cultivars from Italian germplasm. *Plant Mol. Biol. Rep*. 33, 458–473. doi: 10.1007/s11105-014-0754-9

Liebhard, R., Gianfranceschi, L., Koller, B., Ryder, C. D., Tarchini, R., Van De Weg, E., et al. (2002). Development and characterisation of 140 new microsatellites in apple (*Malus x domestica* Borkh.). *Mol. Breed.* 10, 217–241. doi: 10.1023/A:1020525906332

Liebhard, R., Koller, B., Gianfranceschi, L., and Gessler, C. (2003). Creating a saturated reference map for the apple (*Malus* × *domestica* Borkh.) genome. *Theor. Appl. Genet.* 106, 1497–1508. doi: 10.1007/s00122-003-1209-0

Liu, Z., Bao, D., Liu, D., Zhang, Y., Ashraf, M. A., and Chen, X. (2016). Construction of a genetic linkage map and QTL analysis of fruit-related traits in an F1 Red Fuji × Hongrou apple hybrid. *Open Life Sci.* 11, 487–497. doi: 10.1515/biol-2016-0063

Longinelli, A., and Selmo, E. (2003). Isotopic composition of precipitation in Italy: a first overall map. *J. Hydrol.* 270, 75–88. doi: 10.1016/S0022-1694(02)00281-0

Maliepaard, C., Alston, F. H., van Arkel, G., Brown, L. M., Chevreau, E., Dunemann, F., et al. (1998). Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. *Theor. Appl. Genet.* 97, 60–73. doi: 10.1007/s001220050867

Meagher, T. R., and Thompson, E. (1986). The relationship between single parent and parent pair genetic likelihoods in genealogy reconstruction. *Theor. Popul. Biol.* 29, 87–106. doi: 10.1016/0040-5809(86)90006-7

Meirmans, P., and Van Tienderen, P. (2004). Genotype and GenoDive: two programs for the analysis of genetic diversity of asexual organisms. *Mol. Ecol. Notes* 4, 792–794. doi: 10.1111/j.1471-8286.2004.00770.x

Michalakis, Y., and Excoffier, L. (1996). A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics* 142, 1061–1064.

Miranda, C., Urrestarazu, J., Santesteban, L. G., and Royo, J. B. (2010). Genetic diversity and structure in a collection of ancient Spanish pear cultivars assessed by microsatellite markers. *J. Am. Soc. Hortic. Sci.* 135, 428–437.

Molon, G. (1901). *Pomologia*. Milan: Hoepli.

Moriya, S., Iwanami, H., Yamamoto, T., and Abe, K. (2011). A practical method for apple cultivar identification and parent-offspring analysis using simple sequence repeat markers. *Euphytica* 177, 135–150. doi: 10.1007/s10681-010-0295-8

Mousavi, S., Mariotti, R., Regni, L., Nasini, L., Bufacchi, M., Pandolfi, S., et al. (2017). The first molecular identification of an olive collection applying standard simple sequence repeats and novel expressed sequence tag markers. *Front. Plant Sci.* 8:1283. doi: 10.3389/fpls.2017.01283

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89, 583–590.

Noiton, D. A. M., and Alspach, P. A. (1996). Founding clones, inbreeding, coancestry, and status number of modern apple cultivars. *J. Am. Soc. Hortic. Sci.* 121, 773–782.

Pasquale, G. A. (1876). *Manuale di Arboricoltura da Servire pe' Proprietarii, Agricoltori, Orticoltori, Ingegneri, ecc*. Charleston, SC: Nabu Press.

Patzak, J., Paprštein, F., Henychová, A., and Sedlák, J. (2012). Comparison of genetic diversity structure analyses of SSR molecular marker data within apple (*Malus* × *domestica*) genetic resources. *Genome* 55, 1–19. doi: 10.1139/g2012-054

Pereira-Lorenzo, S., Ramos-Cabrer, A. M., and Dıaz-Hernandez, M. B. (2007). Evaluation of genetic identity and variation of local apple cultivars (*Malus domestica* Borkh.) from Spain using microsatellite markers. *Genet. Resour. Crop Evol.* 54, 405–420. doi: 10.1007/s10722-006-0003-7

Pereira-Lorenzo, S., Ramos-Cabrer, A. M., Gonzalez-Diaz, A. J., and Diaz-Hernandez, M. B. (2008). Genetic assessment of local apple cultivars from La Palma Spain, using simple sequence repeats (SSRs). *Sci. Hortic.* 117, 160–166. doi: 10.1016/j.scienta.2008.03.033

Pereira-Lorenzo, S., Urrestarazu, J., Ramos-Cabrer, A. M., Miranda, C., Pina, A., and Dapena, E. (2017). Analysis of the genetic diversity and structure of the Spanish apple genetic resources suggests the existence of an Iberian genepool. *Ann. Appl. Biol.* 171, 424–440. doi: 10.1111/aab.12385

Pérez-Romero, L. F., Suárez, M. P., Dapena, E., and Rallo, P. (2015). Molecular and morphological characterization of local apple cultivars in Southern Spain. *Genet. Mol. Res.* 14, 1487–1501. doi: 10.4238/2015.February.20.4

Pineau, B., Barbe, J. C., Van Leeuwen, C., and Dubourdieu, D. (2007). Which impact for β-damascenone on red wines aroma? *J. Agric. Food Chem.* 55, 4103–4108. doi: 10.1021/jf070120r

Plotto, A., and McDaniel, M. R. (2000). Characterization of changes in 'Gala' apple aroma during storage using osme analysis, a gas chromatography-olfactometry technique. *J. Am. Soc. Hortic. Sci.* 125, 714–722.

Pritchard, J. K., Stephens, M., and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.

Ramos-Cabrer, A. M., Pereira-Lorenzo, S., and Diaz-Hernandez, M. B. (2007). Morphology and microsatellites in Spanish apple collections. *J. Hortic. Sci. Biotechnol.* 82, 257–265. doi: 10.1080/14620316.2007.11512227

Silfverberg-Dilworth, E., Besse, S., Paris, R., Belfanti, E., Tartarini, S., Sansavini, S., et al. (2005). Identification of functional apple scab resistance gene promoters. *Theor. Appl. Genet.* 110, 1119–1126. doi: 10.1007/s00122-005-1940-9

Silfverberg-Dilworth, E., Matasci, C., Van de Weg, W., Van Kaauwen, M., Walser, M., Kodde, L., et al. (2006). Microsatellite markers spanning the apple (*Malus* × *domestica* Borkh.) genome. *Tree Genet. Genomes* 2, 202–224. doi: 10.1007/s11295-006-0045-1

Sun, R., Li, H., Zhang, Q., Chen, D., Yang, F., Zhao, Y., et al. (2014). Mapping for Quantitative Trait Loci and major genes associated with fresh-cut browning in apple. *HortScience* 49, 25–30.

Tamaro, D. (1929). *Frutta di Grande Reddito*. Milan: Hoepli.

Tonini, S. (1930). *Aumentiamo la Produzione Delle Vecchie Piante da Frutto in Provincia di Perugia. Cattedra Ambulante di Agricoltura*. Perugia: Santucci ed.

Urrestarazu, J., Denancé, C., Miranda, C., Ravon, E., Guyader, A., Guisnel, R., et al. (2016). Analysis of the genetic diversity and structure across a wide range of germplasm reveals prominent gene flow in apple at the European level. *BMC Plant Biol.* 16:130. doi: 10.1186/s12870-016-0818-0

Urrestarazu, J., Miranda, C., Santesteban, L. G., and Royo, B. (2012). Genetic diversity and structure of local apple cultivars from Northeastern Spain assessed by micro satellite markers. *Tree Genet. Genomes* 8, 1163–1180. doi: 10.1007/s11295-012-0502-y

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., and Shipley, P. (2004). Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4, 535–538. doi: 10.1111/j.1471-8286.2004.00684.x

van Treuren, R., Kemp, H., Ernsting, G., Jongejans, B., Houtman, H., and Visser, L. (2010). Microsatellite genotyping of apple (*Malus* × *domestica* Borkh.) genetic resources in the Netherlands: application in collection management and variety identification. *Genet. Resour. Crop Evol.* 57, 853–865. doi: 10.1007/s10722-009-9525-0

Vanderzande, S., Micheletti, D., Troggio, M., Davey, M. W., and Keulemans, J. (2017). Genetic diversity, population structure, and linkage disequilibrium of elite and local apple accessions from Belgium using the IRSC array. *Tree Genet. Genomes* 13:125. doi: 10.1007/s11295-017-1206-0

Vavilov, N. I. (1926). *Centry Proischozdenija Kul'turnych Rastenij (On the origin of cultivated plants)*. Mainz: Gutenberg.

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., et al. (2010). The genome of the domesticated apple (*Malus* × *domestica* Borkh.). *Nat. Genet.* 42, 833–841. doi: 10.1038/ng.654

Vinatzer, B. A., Patocchi, A., Tartarini, S., Gianfranceschi, L., Sansavini, S., and Gessler, C. (2004). Isolation of two microsatellite markers from BAC clones of the Vf scab resistance region and molecular characterization of scab-resistant accessions in *Malus* germplasm. *Plant Breed.* 123, 321–326. doi: 10.1111/j.1439-0523.2004.00973.x

Visser, T., and Verhaegh, J. J. (1978). Inheritance and selection of some fruit characters of apple. I. Inheritance of low and high acidity. *Euphytica* 27, 753–760. doi: 10.1007/BF00023711

Waits, L. P., Luikart, G., and Taberlet, P. (2001). Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Mol. Ecol.* 10, 249–256. doi: 10.1046/j.1365-294X.2001.01185.x

WAPA (2017). *Crop Forecast*. Available at: http://www.wapa-association.org/asp/page_1.asp?doc_id=447

Ward, J. H. (1963). Hierarchical grouping to optimize an objective function. *J. Am. Stat. Assoc.* 58, 236–244. doi: 10.1080/01621459.1963.10500845

Weir, B. S., and Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* 38, 1358–1370. doi: 10.2307/2408641

Wright, S. (1978). *Variability Within and Among Natural Populations in Evolution and the Genetics of Populations*, Vol. 4, Chicago, IL: University of Chicago Press.

Wu, J., Gao, H., Zhao, L., Liao, X., Chen, F., Wang, Z., et al. (2007). Chemical compositional characterization of some apple cultivars. *Food Chem.* 103, 88–93. doi: 10.1016/j.foodchem.2006.07.030

Xu, K., Wang, A., and Brown, S. (2012). Genetic characterization of the Ma locus with pH and titratable acidity in apple. *Mol. Breed.* 30, 899–912. doi: 10.1007/s11032-011-9674-7

Yun, W. H., Ban, S. H., Kim, G. H., Kim, J.-H., Kwon, S. I., and Choi, C. (2015). Assessment of apple core collections constructed using phenotypic and genotypic data. *Genet. Mol. Res.* 14, 6453–6464. doi: 10.4238/2015.June.11.21

Zhang, P., Li, J., Li, X., Liu, X., Zhao, X., and Lu, Y. (2011). Population structure and genetic diversity in a rice core collection (*Oryza sativa* L.) investigated with SSR markers. *PLoS One* 6:e27565. doi: 10.1371/journal.pone.0027565

Zhang, Y., Li, P., and Cheng, L. (2010). Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in 'Honeycrisp' apple flesh. *Food Chem.* 123, 1013–1018. doi: 10.1016/j.foodchem.2010.05.053

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership