NEW ROOTSTOCKS FOR FRUIT CROPS: BREEDING PROGRAMS, CURRENT USE, FUTURE POTENTIAL, CHALLENGES AND ALTERNATIVE STRATEGIES

EDITED BY: Sergio Ruffo Roberto, Vittorino Novello and Gennaro Fazio

PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-923-2
DOI 10.3389/978-2-88974-923-2

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: frontiersin.org/about/contact

NEW ROOTSTOCKS FOR FRUIT CROPS: BREEDING PROGRAMS, CURRENT USE, FUTURE POTENTIAL, CHALLENGES AND ALTERNATIVE STRATEGIES

Topic Editors:
Sergio Ruffo Roberto, State University of Londrina, Brazil
Vittorino Novello, University of Turin, Italy
Gennaro Fazio, Plant Genetic Resources Unit (USDA ARS), United States

Citation: Roberto, S. R., Novello, V., Fazio, G., eds. (2022). New Rootstocks for Fruit Crops: Breeding Programs, Current Use, Future Potential, Challenges and Alternative Strategies. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-923-2

Table of Contents

frontiers in Plant Science

EDITORIAL
published: 23 March 2022
doi: 10.3389/fpls.2022.878863

Editorial: New Rootstocks for Fruit Crops: Breeding Programs, Current Use, Future Potential, Challenges and Alternative Strategies

Sergio Ruffo Roberto[1]*, *Vittorino Novello*[2]* and *Gennaro Fazio*[3]*

[1] Department of Agronomy, State University of Londrina, Londrina, Brazil, [2] Dipartimento di Scienze Agrarie, Forestali ed Alimentari, University of Turin, Turin, Italy, [3] Plant Genetic Resources Unit, USDA/ARS, Geneva, NY, United States

Keywords: rootstocks, fruit, breeding, scion compatibility, breeding programs

Editorial on the Research Topic

New Rootstocks for Fruit Crops: Breeding Programs, Current Use, Future Potential, Challenges and Alternative Strategies

INTRODUCTION

Rootstocks are playing an increasingly crucial role in determining orchard efficiency and sustainability in fruit crops. Combining the desirable attributes of two or three different individuals by budding or grafting can produce dramatic effects on growth and productivity (Bowman and McCollum, 2015). The effect of rootstocks on fruit quality in terms of physical traits and internal chemical compositions is well known in several fruit crops. Rootstocks can influence precocity/juvenility, yield, tree size control, biotic and abiotic stress resistance or tolerance, fruit respiratory behavior, crop load and canopy management techniques (Domingues et al., 2021).

There has been major progress made by rootstock breeders in the second half of the last century and the beginning of the present century. The increased breeding activity of rootstock breeders is the reason why a wide range of new rootstocks are available to fruit growers. However, breeding rootstocks for fruit crops is much slower than scion breeding within the same species (Cousins, 2005). This is due to the long testing requirements of rootstocks that reduce the opportunity for comprehensive first stage tests on individual plants compounded by expanding selection criteria for new rootstocks. It is much easier to re-graft a scion than replant an orchard.

The current global agricultural challenges imply the need to generate new technologies and farming systems to cope with the need for sustainability and to face up to climate change. In this context, rootstocks are an essential component for fruit crops in modern agriculture. Currently most rootstocks used are clonally propagated and there are several ongoing efforts to develop these plant materials (Gainza et al., 2015). The aim of this Research Topic was to present the latest results of new rootstocks developed using classic and modern selection techniques and forecast novel applications.

In this context, Rufato et al. examined productive performance of apple cultivars grafted on selected Geneva® series rootstocks under extreme conditions of apple replant disease (ARD) areas in southern Brazil, including "Gala Select" and "Fuji Suprema" apples (*Malus domestica* [Suckow] Borkh.) grafted on "G.202," "G.210," "G.213," and "G.814" rootstocks. It was found that the non-fallow condition does not alter the relative differences in vigor and apple fruit quality among the rootstocks, and the G.210 semi-dwarfing rootstock is an alternative for the immediate conversion

OPEN ACCESS

Edited and reviewed by:
Claudio Bonghi, University of Padua, Italy

***Correspondence:**
Sergio Ruffo Roberto
sroberto@uel.br
Vittorino Novello
vittorino.novello@unito.it
Gennaro Fazio
Gennaro.Fazio@ars.usda.gov

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

Received: *18 February 2022*
Accepted: *28 February 2022*
Published: *23 March 2022*

Citation:
Roberto SR, Novello V and Fazio G (2022) Editorial: New Rootstocks for Fruit Crops: Breeding Programs, Current Use, Future Potential, Challenges and Alternative Strategies. Front. Plant Sci. 13:878863. doi: 10.3389/fpls.2022.878863

of "Gala Select" and "Fuji Suprema" apple orchards under these conditions. Within the same species and topic, Mao et al. explored the potential ARD resistance of "12-2" elite rootstock selection and compared it with "M.9-T337" and "M.26" rootstocks, which are commonly grown in China. Authors found that "12-2" elite rootstock can be used as an important genetic source material for breeding of ARD-resistant apple rootstocks, which will be essential for fundamentally solving the rampant problem of ARD in China.

Moving toward another important tree crop species, i.e., citrus (*Citrus sinensis* L.), Bowman et al. described the USDA's citrus breeding program novel, multi-pronged, strategy termed "SuperSour," for rootstock breeding and presented its key components and methodologies, along with reference to the historical favorite rootstock sour orange (*Citrus aurantium*), and previous methods employed in citrus rootstock breeding. One of characteristics of this strategy is the rootstock propagation by cuttings and or *in-vitro* methods which avoid the need for nucellar seeds (and the associated juvenility period), increases testing replication and eliminates a 6- to 15-year delay in testing while waiting for new hybrids to fruit. As a result, many of the new "SuperSour" hybrid rootstocks exhibited greatly superior fruit yield, yield efficiency, canopy health, and fruit quality, as compared with the standard rootstocks. Within the same species, Carvalho et al. investigated the effects of fruit maturity on seed quality and seedling performance of "US-802," "US-897," and "US-942" citrus rootstocks in Florida, US, including the evaluation of seed germination and nursery performance of the seedlings. Authors found that fruit from all three rootstock varieties can be harvested as early as August without losing any germination potential. In another trial, Cruz et al. evaluated the influence of five rootstocks on the vegetative growth, yield performance, fruit quality, and HLB tolerance of "Emperor" mandarin (*Citrus reticulata* Blanco) under the Southern Brazilian humid subtropical climate. Based on their findings "Cleopatra" mandarin, "Sunki" mandarin, "Swingle" citrumelo, and "Fepagro C-13" citrange were considered more suitable rootstocks for "Emperor" mandarin under such conditions.

Some interesting aspects of grapevine (*Vitis* spp.) rootstocks regarding their tolerance to fungal grapevine trunk diseases (GTDs) were investigated by Ramsing et al. in Spain. Twenty-five rootstocks were screened for xylem characteristics and tolerance to main associated fungi. Authors found differences in all the analyzed xylem traits, and also in DNA concentration for both of the main associated fungi among the tested rootstocks. This finding is an important tool to support future rootstock breeding programs to reduce the detrimental impact of GTDs worldwide.

The rootstock-mediated genetic contributions in recombinant juvenile cacao (*Theobroma cacao* L.) across target traits, specifically cadmium (Cd) uptake, and its correlation with growth and physiological traits, were addressed by Férnandez-Paz et al., in which 320 progenies were used as rootstocks in grafts with two commercial clones (ICS95 and CCN51) commonly grown in Colombia. Authors found that differences in the specific combining ability for Cd uptake were mostly detected in ungrafted rootstocks, or 2 months after grafting with the clonal CCN51 scion. These findings will harness early breeding schemes of cacao rootstock genotypes compatible with commercial clonal scions and adapted to soils enriched with toxic levels of Cd.

Also in Colombia, Cañas et al. assessed how elite "crioulo" "plus trees" of avocado rootstocks (*Persea americana* Mill.) inherit trait variation to their seedling progenies, and whether such family superiority may be transferred after grafting to the clonal scion. The results revealed that that elite "criollo" "plus trees" may serve as promissory donors of seedling rootstocks for avocado cv. Hass due to the inheritance of their outstanding trait values.

Finally, Xiong et al. evaluated in China the graft compatibility of melon cv. Akekekouqi (*Cucumis melo*) grafted onto eight *Cucurbitaceae* species including cucumber, pumpkin, melon, luffa, wax gourd, bottle gourd, bitter gourd, and watermelon. The starch–iodine staining technique was used to predict graft compatibility. Authors found that cucumber and pumpkin are graft compatible with melon, while luffa, wax gourd, bottle gourd, bitter gourd, and watermelon are graft incompatible. Also, it was demonstrated that graft compatibility can be evaluated earlier by the starch–iodine staining technique, supporting breeding programs.

AUTHOR CONTRIBUTIONS

SR, VN, and GF: writing—review and editing. All authors contributed to the article and approved the submitted version.

REFERENCES

Bowman, K. D., and McCollum, G. (2015). Five new citrus rootstocks with improved tolerance to huanglongbing. *HortScience* 50, 1731–1734. doi: 10.21273/HORTSCI.50.11.1731

Cousins, P. (2005). Rootstocks breeding: an analysis of intractability. *HortScience* 40, 1945–1946. doi: 10.21273/HORTSCI.40.7.1945

Domingues, A. R., Marcolini, C. D. M., Gonçalves, C. H., Resende, J. T. V., Roberto, S. R., and Carlos, E. F. (2021). Rootstocks genotypes impact on tree development and industrial properties of 'Valencia' sweet orange juice. *Horticulturae* 7, e-141. doi: 10.3390/horticulturae7060141

Gainza, F., Opazo, I., Guajardo, V., Meza, P., Ortiz, M., Pinochet, J., Muñoz, C. (2015). Rootstock breeding in *Prunus* species: ongoing efforts and new challenges. *Chilean J. Agric. Res.* 75, 6–16. doi: 10.4067/S0718-58392015000300002

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 August 2021
doi: 10.3389/fpls.2021.712162

Geneva® Series Rootstocks for Apple Trees Under Extreme Replanting Conditions in Southern Brazil

Leo Rufato[1], Pricila Santos da Silva[1], Aike Anneliese Kretzschmar[1], Amauri Bogo[1], Tiago Afonso de Macedo[1], Juliana Fátima Welter[1], Gennaro Fazio[2] and Daiana Petry[3]*

[1] Department of Agronomy, University of Santa Catarina State, Florianópolis, Brazil, [2] U.S. Department of Agriculture - Agricultural Research Service (USDA-ARS), Plant Genetic Resources Unit Geneva, Geneva, NY, United States, [3] Department of Environmental Engineering, University of Santa Catarina State, Florianópolis, Brazil

Geneva® rootstocks in Brazil are known to be efficient in controlling vigor, and are precocious and resistant to diseases. The objective of this study was to evaluate the performance of apple tree cultivars grafted on the Geneva® rootstocks in severe replant disease areas, by planting 60 days after the eradication. The experiments were implemented in 2017, in São Joaquim and Vacaria. The Gala Select and Fuji Suprema cultivars were grafted onto 'G.202', 'G.814', 'G.210', and 'G.213' rootstocks in the Tall Spindle training system. In 2018/2019, total thinning was carried out to promote plant growth. In São Joaquim, partial thinning was carried out in 2019/2020 harvest of 'Gala Select'. The rootstocks were divided into two groups based on vigor, for both areas and cultivars. 'G.202' and 'G.213' were 40% less vigorous than 'G.210' and 'G.814'. For 'Gala Select', the extreme non-fallow condition mainly affected the vigor and productivity of 'G.213' in both areas. At the end of two harvests, 'G.213' was 17% less productive than 'G.210', contrary to what is observed in areas where the fallow period is respected. However, 'G.213' confirmed a greater yield efficiency, which was 27% higher than 'G.210'. This suggests that a perspective of forecasting production for the third crop is higher for 'G.213' than for 'G.210'. In the case of 'Fuji Suprema', the G.210 rootstock was the most productive in both areas. In São Joaquim, 'G.202' matched 'G.210' in productivity and efficiency as it sprouts better in colder regions. Considering the fruit quality, 'G.213' anticipated the maturation with fruits of larger size and higher total soluble solids (TSS) in both areas and cultivars, making it possible to anticipate the harvest. It was concluded that the non-fallow condition does not alter the relative differences in vigor and fruit quality among the rootstocks. However, notwithstanding the overall replant tolerance of these rootstocks, it does reduce productivity by mainly affecting less vigorous rootstocks that need about three crops to overcome the allelopathic effects of the soil and start growing normally. The G.210 semi-dwarfing rootstock is an alternative for the immediate conversion of apple orchards of Gala Select and Fuji Suprema cultivars in southern Brazil.

Keywords: apple tree, yield efficiency, replanting area, 'G.213', 'G.210'

OPEN ACCESS

Edited by:
Claudio Bonghi, University of Padua, Italy

Reviewed by:
Poliana Francescatto, Valent BioSciences LLC, United States
Carlos Danilo Cabrera Bologna, National Institute for Agricultural Research (INIA), Uruguay

****Correspondence:***
Leo Rufato
leoruffato@yahoo.com.br

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

Received: *20 May 2021*
Accepted: *02 August 2021*
Published: *30 August 2021*

Citation:
Rufato L, da Silva PS, Kretzschmar AA, Bogo A, de Macedo TA, Welter JF, Fazio G and Petry D (2021) Geneva® Series Rootstocks for Apple Trees Under Extreme Replanting Conditions in Southern Brazil. Front. Plant Sci. 12:712162. doi: 10.3389/fpls.2021.712162

INTRODUCTION

Most of the modern apple orchards rely on dwarfing rootstocks, which produce a more compact tree allowing for high density planting and earlier and higher yields, and therefore, great economic viability (Afonso et al., 2017). Anticipated production, alternate bearing (Kviklys et al., 2016), resistance and tolerance to pests and diseases (Beers et al., 2006), drought resistance capacity (Tworkoski and Fazio, 2016), resistance to sprouting time, sensory characteristics, and physicochemical composition of fruits (Kviklys et al., 2015) are the other characteristics of apple trees induced by the rootstocks or by the combination of the canopy cultivar and the rootstocks (Harrison et al., 2016).

Currently, in Brazil, there is a gradual change toward the use of densely packed apple orchards with the introduction of the Geneva® series rootstocks (e.g., 'G.210' and 'G.213') in commercial areas of large companies such as Fischer, Hiragami, Rasip, and Schio. The Geneva® series rootstocks were developed by the apple rootstock breeding program at Cornell University with the original intention of breeding rootstocks resistant to fire blight, collar rot, wooly apple aphid, and replant disorders in addition to size control (Robinson et al., 2011; Fazio et al., 2012). These rootstocks have a wide vigor range, allowing greater plant densities per area and better light inside the canopy (Fazio et al., 2013).

In the south of Brazil, some research has been carried out with the Geneva® series rootstocks 'G.202' 'G.213', 'G.210', and 'G.814'. According to Denardi et al. (2015), 'G.213' has good adaptability and production stability, whereas the G.202 rootstock has less constant productivity over the years. These same authors classified G.202 and G.213 rootstocks as dwarves (vigor similar to M.9). According to Macedo et al. (2019), the Fuji Suprema cultivar grafted on 'G.213' is more productive in both virgin and replanting soil. The G.210 rootstock is considered semi-dwarf (Denardi et al., 2015) and has vigor similar to 'G.814'. According to Pasa et al. (2016), the G.814 rootstock has the potential for use in high-density orchards.

Before choosing the most adapted rootstock, the capabilities and limitations of each rootstock should be evaluated in each growing condition (Gjamovski and Kiprijanovski, 2011). Thus, the objective of this work was to evaluate the productive performance of apple cultivars grafted on the Geneva® series rootstocks under extreme conditions of replanting areas in southern Brazil.

MATERIALS AND METHODS

Two experiments with the 'Gala Select' and 'Fuji Suprema' grafted on Geneva® rootstocks 'G.202', 'G.210', 'G.213', and 'G.814' were implemented.

The first experiment was implemented in a commercial orchard of the company named Mareli, located at an altitude of 1,364 m, with the geographic coordinates of 28°16' south latitude and 49°56' west longitude, in São Joaquim, Santa Catarina, Brazil. The soils of the region fall into the classes Cambisolo Humico, Neossolo Litólico, and Nitossolo Háplico, formed from riodacite rock and basalt (Embrapa, 2004). The climate is humid mesothermal type with mild summers, which is Cfb according to the classification of Köppen (Köppen, 1948).

The second experiment was implemented in a commercial orchard of the company Agropecuária Schio Ltda, located at an altitude of 971 m, with the geographical coordinates of 28°24' south latitude and 50°50' west longitude, in Vacaria, Rio Grande do Sul. The climate according to the Köppen classification is Cfb, subtropical with mild summers (Köppen, 1948). The maximum, minimum, and average air temperatures have a marked annual amplitude (Pereira et al., 2009). The soils of the region can be classified as Latossolo Bruno, with smooth to wavy relief, containing high levels of clay and aluminum (Embrapa, 2004).

Both the experiments were implemented in 2017, about 60 days after the eradication of the old orchard, under extreme replanting conditions with no fallow period before planting the trials. The spacing between trees and rows, pruning, training system, and thinning adopted were based on the current grower preference for each region.

In 2018/2019, all fruits were removed during the spring from both the varieties and regions to encourage vegetative growth. In São Joaquim area, in the winter of 2019, pruning was carried out to form and train the trees in the two-dimensional fruit wall system. Partial thinning was carried out in the 2019/2020 harvest, aiming to standardize the production to approximately 7 $t.ha^{-1}$ or 1 (one) fruit per cluster. In Vacaria, in the winter of 2019, minimal pruning was carried out with the sole aim of removing branches that were competitive with the leader centering and training of the trees by the tall spindle system. Maximum plant production was prioritized, with no thinning in 2019/2020. In 2020/2021, no thinning was carried out in both areas.

In São Joaquim area, the trial was planted with a spacing of 0.90 m between trees and 3.2 m between rows (3,472 $trees.ha^{-1}$) for 'Gala Select'; 1.0 m between trees and 3.2 m between rows (3,125 $trees.ha^{-1}$) for 'Fuji Suprema'. In Vacaria area, the trial was planted with a spacing of 0.90 m between trees and 4.0 m between rows (2,777 $trees.ha^{-1}$) for 'Gala Select'; 1.0 m between trees and 4.0 m between rows (2,500 $trees.ha^{-1}$) for 'Fuji Suprema'.

In 2018/2019, all fruits were removed to encourage vegetative growth. Thus, the analyses accounted for 3 years of vegetative data and two consecutive crops of yield performance and fruit quality. Trunk cross-sectional area (TCSA) (cm^2), total plant height (m), TCSA increase (cm^2), canopy volume (m^3), number of branches, cumulative yield ($t.ha^{-1}$), cumulative yield efficiency ($kg.cm^{-2}$), fruit size (100–120 mm, 120–150 mm, or higher than 150 mm), firmness (*N*), and total soluble solids (TSS) (°Brix) were measured. In both the experiments, we adopted the following methods to measure these variables:

- Trunk cross-sectional area (TCSA) (cm^2): it was obtained by averaging the longitudinal and transversal measurements of trunk diameter planting line, 10 cm above the grafting point. To transform the diameter values into TCSA, the equation $A = (\pi d^2)/4$ was used, where d = trunk diameter.

- Total plant height (m): it was measured with a topographic ruler, from the grafting point to the apex of the plant.
- TCSA increase (cm^2): the average increase in TCSA was obtained by calculating the average of the differences between subsequent years.
- Canopy volume (m^3): obtained by measuring L = length, H = height from first branch insertion point to apex, W = width, and using equation (L × H × W) (Macedo et al., 2019).
- Number of branches: it was obtained by counting all the branches larger than 10 cm spreading out from the central leader.
- Cumulative yield ($t.ha^{-1}$): it was obtained by adding the yield of all harvests.
- Cumulative yield efficiency ($kg.cm^{-2}$): it was obtained by adding the yield efficiency of all harvests. The yield efficiency was calculated through the ratio of fruit weight mean per plant ($kg.plant^{-1}$) to crown trunk cross-sectional area (cm^2), expressed in kg of fruits produced per square centimeter of TCSA.
- Fruit size: a sample of 20 fruits per plot was used to classify the size of the fruits. A ruler with circular holes of different diameters was used to measure the fruits and classify them into three sizes: 100–120 mm (low size), 120–150 mm (intermediate size), and >150 mm (high size). The results were expressed as percentage.
- Firmness (*N*): a sample of 50 fruits per plot was measured with a digital texturometer with 11 mm tip. The measurement was performed in the fruit equatorial zone, after two epidermis discs of about 1 cm in diameter were removed from opposite sides.
- Total Soluble Solids (TSS) (°Brix): it was determined with a digital refractometer from the juice extracted from a slice of each fruit in a sample of 50 fruits.

The experiments were implemented in randomized blocks, using four blocks, four treatments, and five replications. The data were analyzed using univariate and multivariate methods. In the univariate method, they were submitted to F-test and when significant, analyzed by Tukey's test ($p \leq 0.05$). Before ANOVA, the Shapiro–Wilk and Bartlett tests were performed to analyze normality and homogeneity of variances. Multivariate analysis was performed using principal component analysis (PCA) to analyze the interrelation between variables.

RESULTS

The results were presented based on two stages. The first stage evaluated the productivity and yield efficiency progression over time. The second stage evaluated the vigor-productivity-efficiency relationship using the final averages of the experiments.

Area of São Joaquim

In the first harvest (2020), 'Gala Select' grafted on 'G.814' and 'G.210' was more productive than those grafted on other rootstocks. In the second year (2021), the trees on G.202 and G.210 rootstocks had higher productivity. Thus, Gala Select cultivar grafted on the G.210 rootstock had higher accumulated productivity ($54\ t.ha^{-1}$) in extreme replanting conditions, 25% higher than the G.213 rootstock ($43\ t.ha^{-1}$) and 14% higher than G.814 and G.202 rootstocks (each $47\ t.ha^{-1}$). The trees with greater yield efficiency were grafted on G.213 rootstock, which was 34% higher than the G.210 rootstock (**Figure 1**).

For the Fuji Suprema cultivar, high yields were observed on G.814 and G.210 rootstocks in 2020 and on G.210 rootstock in 2021 (**Figure 2**). Over the period of these 2 years, 'Fuji Suprema' on the G.210 rootstock was 102% more productive ($77\ t.ha^{-1}$) than on the G.202 rootstock ($38\ t.ha^{-1}$). The results of greatest yield efficiency were observed in 'Fuji Suprema' on G.213 rootstock.

Figures 3, **4** present the principal component analysis results of the vigor-productivity-efficiency relationship of the Gala Select and Fuji Suprema cultivars. The points represent the rootstocks, and the axes represent the vectors that describe the weight of variables to represent the behavior of the first two main components. **Figure 3** shows the results of the Gala Select cultivar and **Figure 4** shows that of the Fuji Suprema cultivar.

In both cultivars, the distancing of the dwarfing rootstocks ('G.202' and 'G.213') from the semi-dwarfing rootstocks ('G.210' and 'G.814') is observed mainly in terms of vigor. The rootstocks 'G.202' and 'G.213' were 38.7 and 40% less vigorous than 'G.210' and 'G.814' in 'Gala Select' and 'Fuji Suprema', respectively.

In the Gala Select cultivar, the dwarfing rootstocks G.202 and G.213 showed the highest yield efficiency (40%). Between G.202 and G.213 rootstocks there was a difference in relation to productivity. 'G.202' correlated with both yield efficiency and productivity, providing greater productivity to the canopy cultivar (11.3%). Despite both being dwarfing rootstocks, 'G.202' was about 16.3% more vigorous and 18% less efficient than 'G.213'. Between the less efficient rootstocks G.210 and G.814, a clear difference in relation to the vigor and productivity of 'Gala Select' is shown in **Figure 3**. 'G.210' was more productive (15.3%) and less vigorous than 'G.814' (9%).

For the Fuji Suprema cultivar, the yield efficiency did not differentiate the dwarfing rootstocks from the semi-dwarfing ones. In this cultivar, the G.202 rootstock proved to be less efficient and less productive compared to all the others −29% less efficient than 'G.213' and 9% less efficient than the semi-dwarfing rootstocks. Thus, 'G.213' maintained the characteristic of greater yield efficiency for both cultivars.

The highest yields of the Fuji Suprema cultivar were on the semi-dwarfing rootstocks G.210 and G.814. In this cultivar, 'G.814' surpassed the productivity of 'G.213' by 23% and was 18% below 'G.210'. **Figure 4** shows a greater correlation between 'G.210' and the vigor variables in the Fuji Suprema cultivar compared to that shown in 'Gala Select' (**Figure 3**). The position of 'G.210' and 'G.814' in relation to the vigor variables indicates the superiority of the G.210 rootstock. In other words, in the Fuji Suprema cultivar in São Joaquim, 'G.210' showed greater vigor (8% ASTT) than 'G.814'.

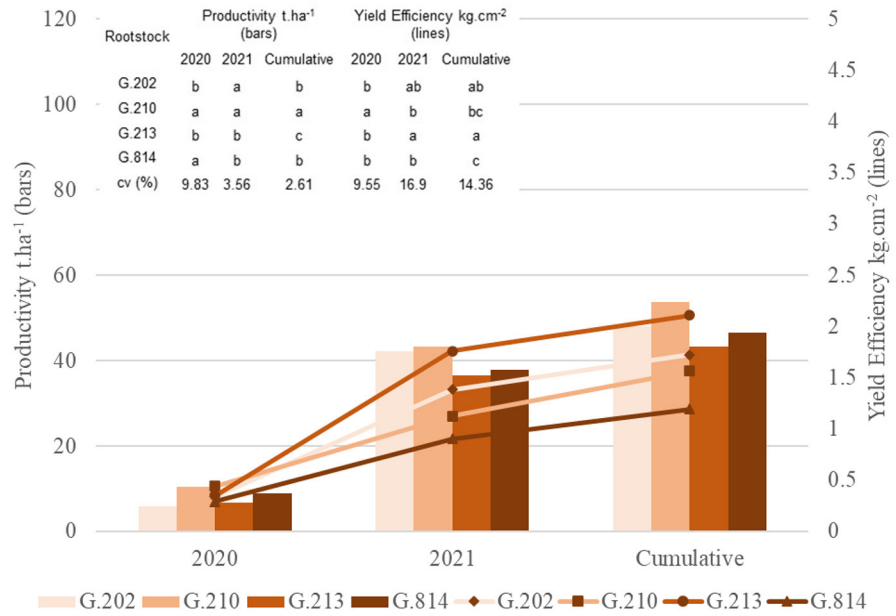

FIGURE 1 | Yield and yield efficiency of the Gala Select cultivar grafted on different rootstocks, under replanting area in São Joaquim. Yield and yield efficiency that do not share a letter are significantly different (Tukey's test, $\alpha = 0.05$).

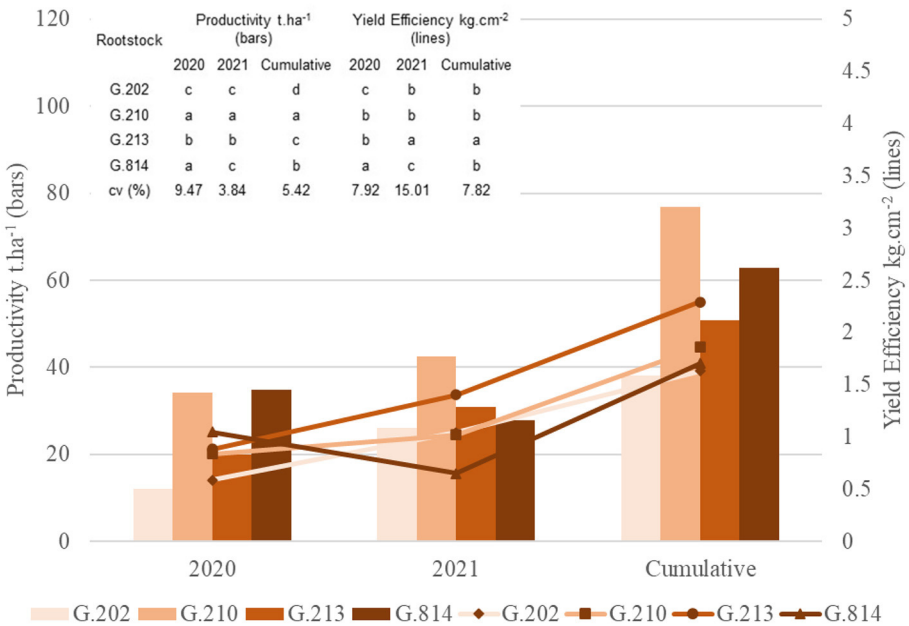

FIGURE 2 | Yield and yield efficiency of the Fuji Suprema cultivar grafted on different rootstocks, under replanting area in São Joaquim. Yield and yield efficiency that do not share a letter are significantly different (Tukey's test, $\alpha = 0.05$).

Figures 5, **6** present the principal component analysis results of the fruit quality dimension, for the Gala Select (**Figure 5**) and Fuji Suprema (**Figure 6**) cultivars.

Regarding the fruit quality parameters of the Fuji Suprema cultivar, the dwarfing rootstocks differed from the semi-dwarfing rootstocks in having a higher index of total soluble solids. The firmness axis was responsible for differentiating between 'G.202' and 'G.213'. 'G.202' showed characteristics of greater fruit firmness than 'G.213'. All rootstocks provided fruits similarly distributed among the three sizes classes. All of them had the

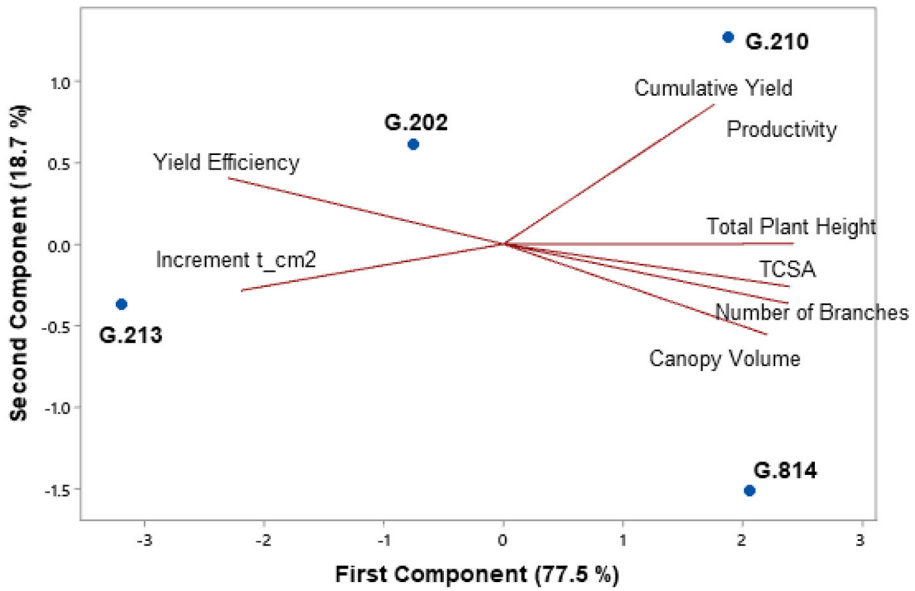

FIGURE 3 | Vigor-productivity-efficiency relationship of the Gala Select cultivar on the Geneva® series rootstocks. São Joaquim – SC.

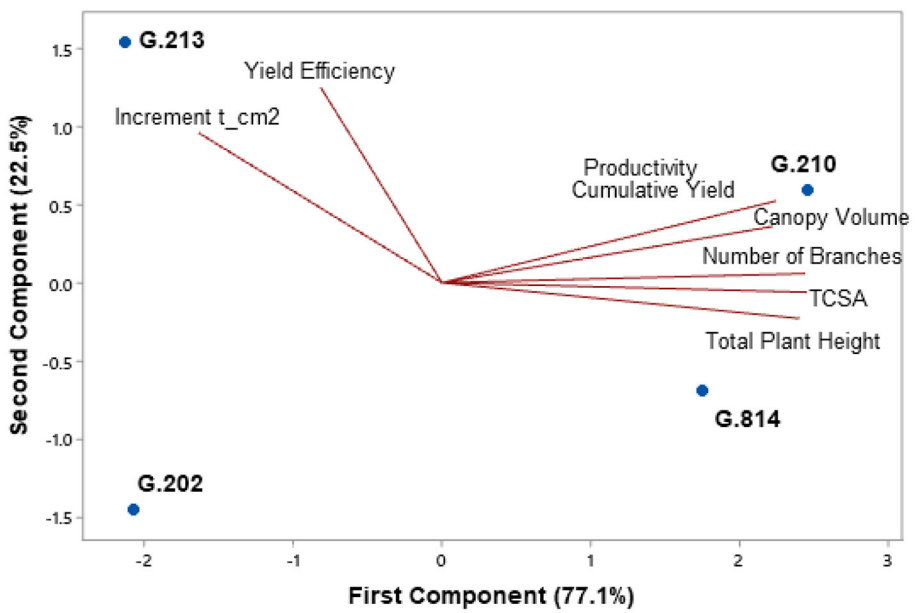

FIGURE 4 | Vigor-productivity-efficiency relationship of the Fuji Suprema cultivar on the Geneva® series rootstocks. São Joaquim – SC.

largest number of fruits categorized in the intermediate size class (100% more than in the low size class and 25% more than in the high size class). However, the position of 'G.210' on the graph (**Figure 6**) shows a greater number of fruits in the high size class and lesser in the low size class compared to the other rootstocks.

For the Gala Select cultivar, all rootstocks provided most of the fruits categorized as high size. However, the positions of 'G.213' and 'G.814' on the graph (**Figure 5**) identify them with higher amounts of high size fruits compared to 'G.202' and 'G.210'. The difference between 'G.213' and 'G.814' is on the axis of the low size class. 'G.213' has a positive correlation with this axis. In other words, 'G.213' has a higher number of fruits categorized as low size when compared to 'G.814'. 'G.202' continued to provide fruits with greater firmness than the other rootstocks. 'G.210' resembled 'G.202' and 'G.213' in total soluble solids.

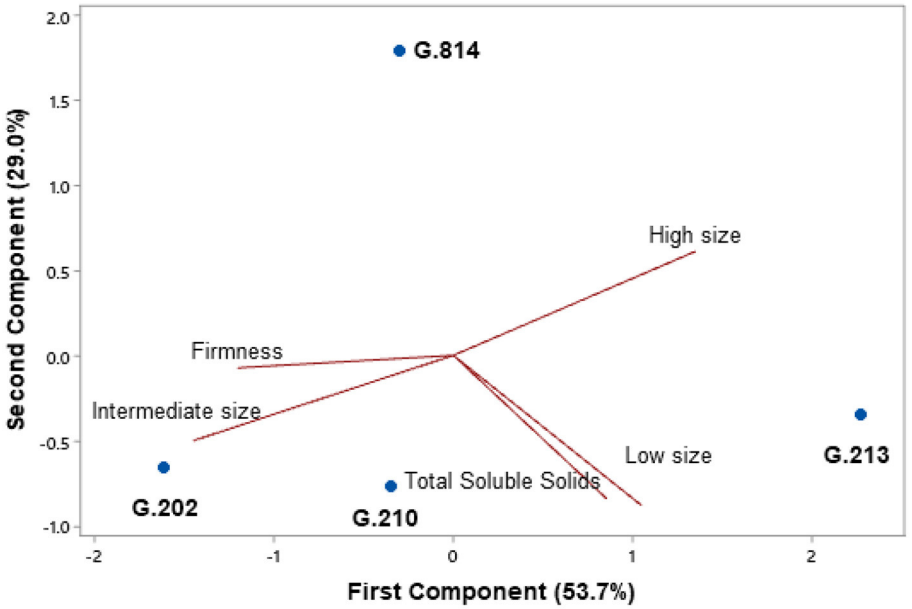

FIGURE 5 | Fruit quality analysis of the Gala Select cultivar on the Geneva® series rootstocks. São Joaquim - SC.

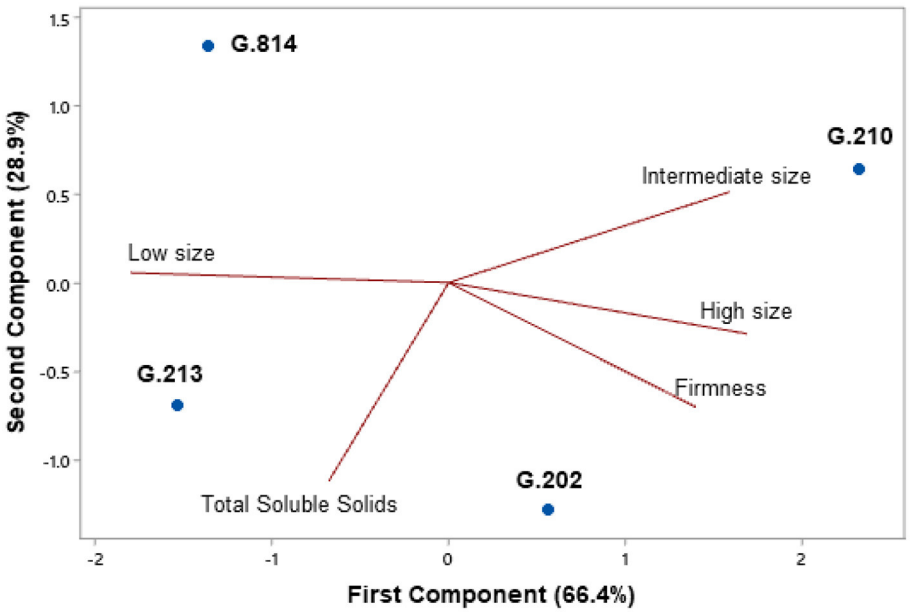

FIGURE 6 | Fruit quality analysis of the Fuji Suprema cultivar on the Geneva® series rootstocks. São Joaquim - SC.

Area of Vacaria

In the first harvest (2020), the Gala Select cultivar was more productive on the G.210 and G.213 rootstocks (**Figure 7**). In the second harvest (2021), 'Gala Select' grafted on 'G.210' had high productivity. In the sum of these 2 years, the Gala Select cultivar on 'G.210' was 143% (56 t.ha^{-1}) higher than 'G.202' (23 t.ha^{-1}) and 24% higher than G.814 and G.213 rootstocks (45 t.ha^{-1}). However, the G.213 rootstock had a higher yield efficiency than the other rootstocks.

The Fuji Suprema cultivar had high productivity when grafted on the G.814 rootstock in the 2020 and 2021 harvests, and on the G.210 rootstock in the 2021 harvest (**Figure 8**). Thus, the cumulative productivity of these

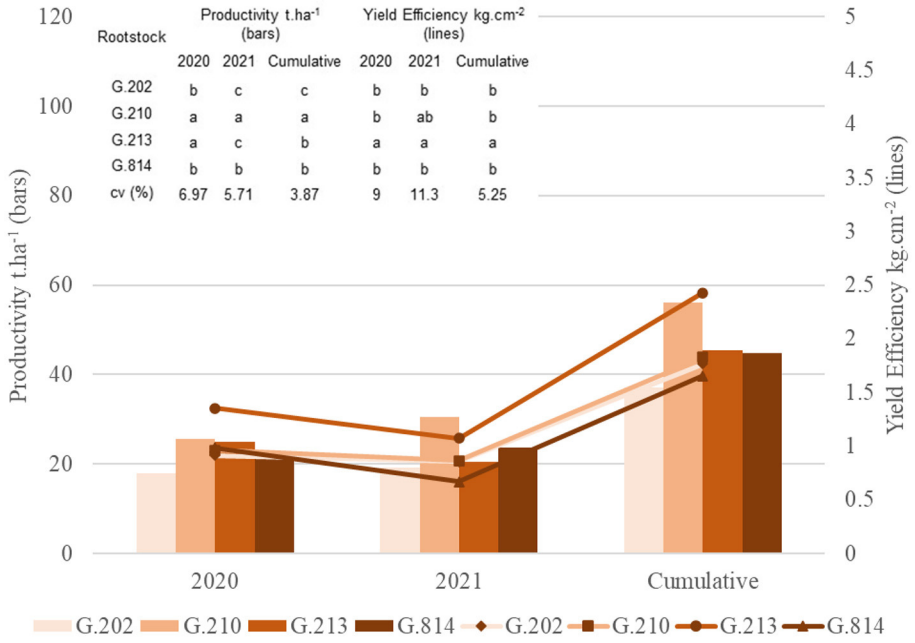

FIGURE 7 | Yield and yield efficiency of the Gala Select cultivar grafted on different rootstocks, under replanting area in Vacaria. Yield and yield efficiency that do not share a letter are significantly different (Tukey's test, $\alpha = 0.05$).

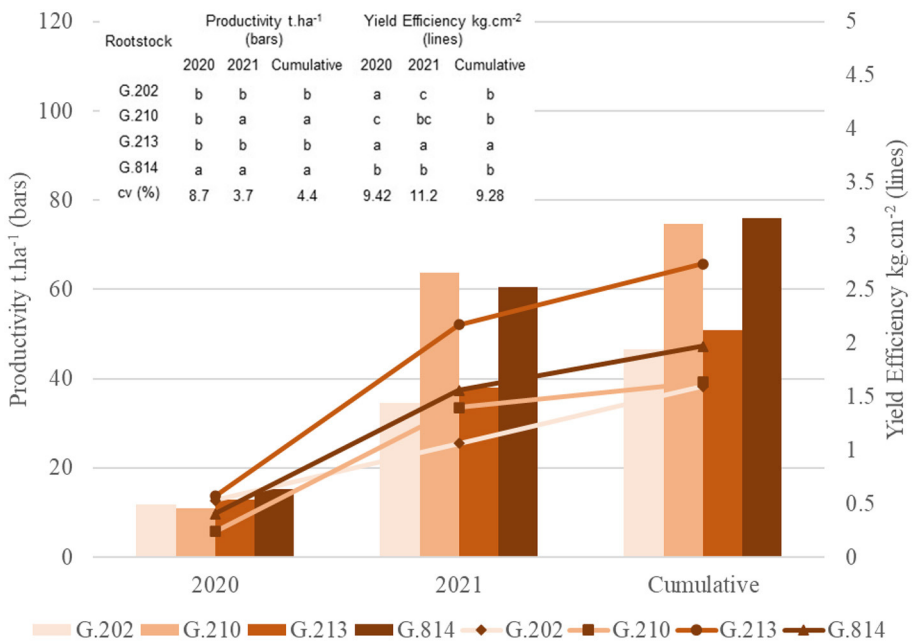

FIGURE 8 | Yield and yield efficiency of the Fuji Suprema cultivar grafted on different rootstocks, under replanting area in Vacaria. Yield and yield efficiency that do not share a letter are significantly different (Tukey's test, $\alpha = 0.05$).

2 years was higher for the G.814 and G.210 rootstocks when compared to the G.202 and G.213 rootstocks. However, even with lower productivity, the trees grafted on 'G.213' had a higher yield efficiency than when grafted on other rootstocks.

Figures 9, **10** present the principal component analysis results of the vigor-productivity-efficiency relationship of the Gala Select and Fuji Suprema cultivars in Vacaria area. The points represent the rootstocks, and the axes represent the vectors that describe the weight of variables to represent the behavior of the first two

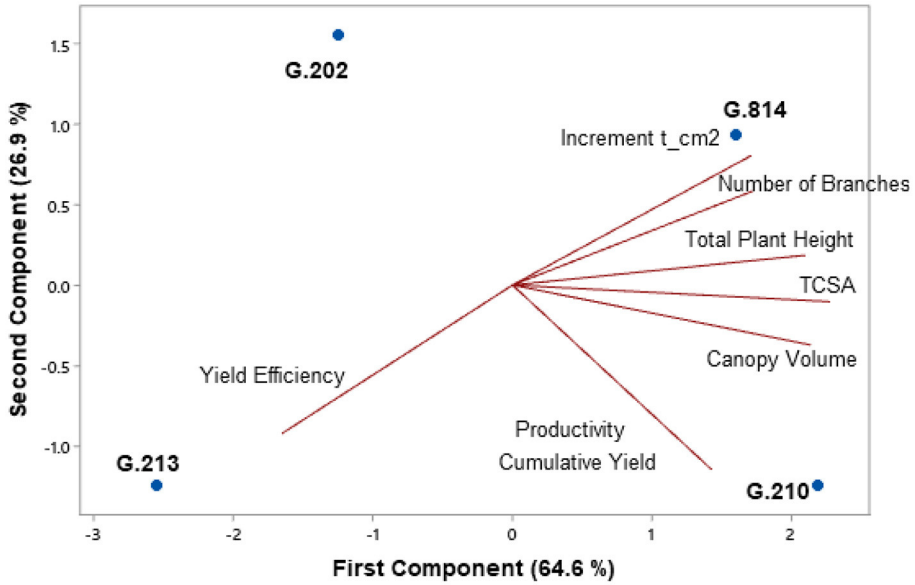

FIGURE 9 | Vigor-Productivity-Efficiency relationship of the Gala Select cultivar on the Geneva® series rootstocks. Vacaria - RS.

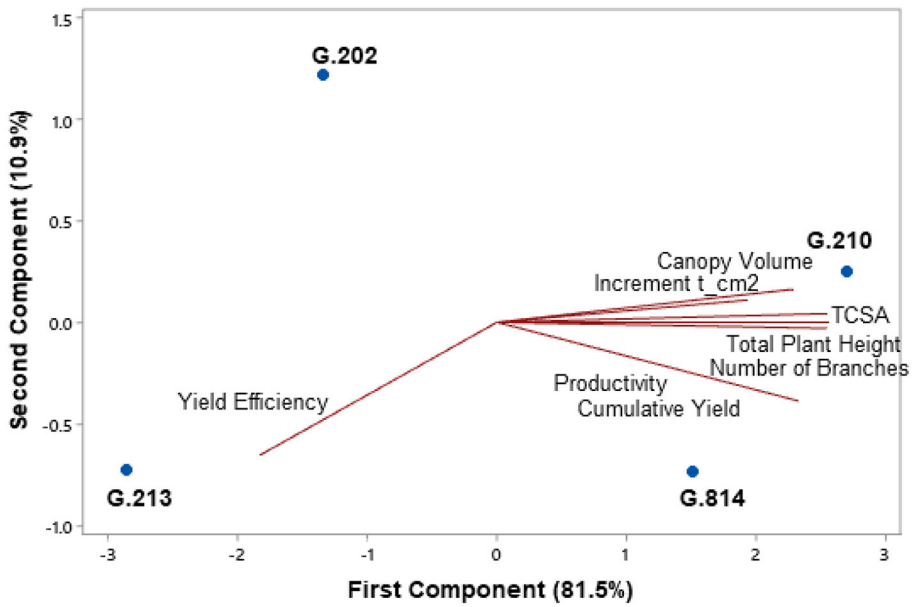

FIGURE 10 | Vigor-productivity-efficiency relationship of the Fuji Suprema cultivar on the Geneva® series rootstocks. Vacaria - RS.

main components. **Figure 9** presents the results of the Gala Select cultivar and **Figure 10** of the Fuji Suprema cultivar.

Based on vigor, there was again the separation of G.814 and G.210 rootstocks from G.202 and G.213 rootstocks for both cultivars. Dwarfing rootstocks were 32 and 43% less vigorous than semi-dwarfing rootstocks for 'Gala Select' and 'Fuji Suprema', respectively.

'G.213' showed higher yield efficiency in both cultivars (43% in 'Gala Select' and 32% in 'Fuji Suprema'). For both cultivars, 'G.202' was characterized by low productivity. For the Fuji Suprema cultivar, the semi-dwarfing rootstocks provided higher yields than the dwarfing ones. The greater vigor induced by these rootstocks did not affect the average productivity, and the Fuji Suprema cultivar grafted on 'G.814' and 'G.210' had 58% higher productivity than when grafted on 'G.202' and 'G.213' ($38 \times 24\,t.ha^{-1}$). However, for the Gala Select cultivar, the productivity vector changed direction separating the G.210 and G.814 rootstocks. The G.210 rootstock continued to show higher

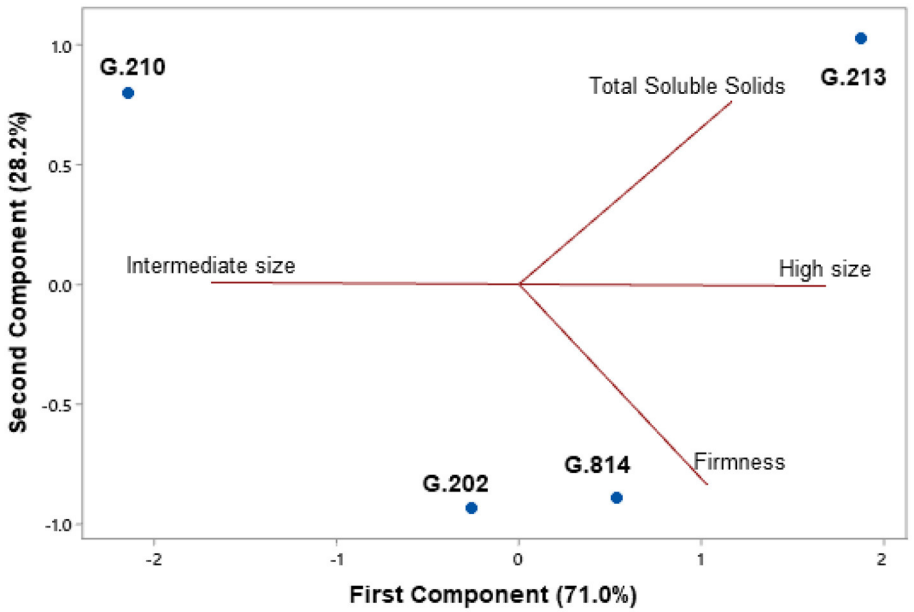

FIGURE 11 | Fruit quality analysis of the Gala Select cultivar on the Geneva® series rootstocks. Vacaria - RS.

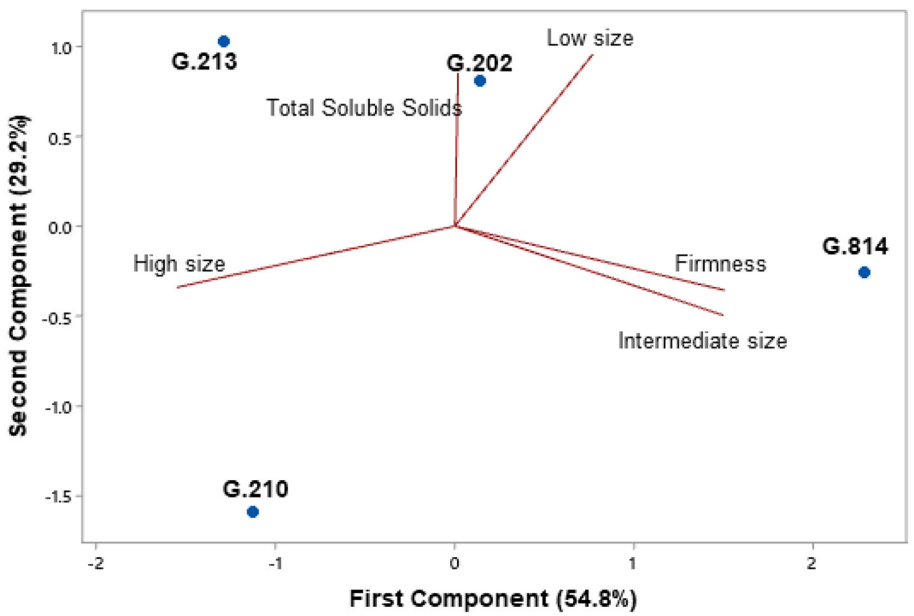

FIGURE 12 | Fruit quality analysis of the Fuji Suprema cultivar on the Geneva® series rootstocks. Vacaria - RS.

productivity, and the yield efficiency of 'G.213' made it equal to 'G.814' in terms of average and accumulated productivity.

Figures 11, **12** present the principal component analysis results of the fruit quality dimension, for the Gala Select (**Figure 11**) and Fuji Suprema (**Figure 12**) cultivars.

Two groups can be identified for the Gala Select cultivars. The G.213 and G.210 rootstocks were characterized by higher total soluble solids and lesser firmness when compared to 'G.202' and 'G.814'. The difference in the positions of 'G.213' and 'G.210' in the graph is due to the fruit size. 'G.213' has a greater number of fruits of high size and a smaller number of fruits of intermediary size when compared to 'G.210'. The G.202 and G.814 rootstocks were characterized by greater firmness and lesser total soluble solids when compared to G.213 and G.210 rootstocks. They were similar in relation to the distribution of fruit size classes, with fruits of high size being greater in quantity than 'G.210' and lesser

than 'G.213'. No rootstock showed fruits of the low-size category for the Gala Select cultivar.

For the Fuji Suprema cultivar, the principal component analysis result was similar to São Joaquim area. The total soluble solids axis differentiated the dwarfing rootstocks from the semi-dwarfing rootstocks. 'G.202' and 'G.213' had higher total soluble solids than 'G.210' and 'G.814'. The G.202 rootstock showed characteristics of greater firmness than the G.213 rootstock. The size and firmness axes were responsible for separating 'G.210' from 'G.814'. The G.210 rootstock provided a larger number of fruits with greater size than the G.814 rootstock. 'G.814' resulted in more firm fruits than 'G.210'. The G.213 rootstock showed a strong positive correlation with the classes of high- and high- and low-size fruits. Thus, the quantity of high-size fruits provided by 'G.213' was similar to that provided by 'G.210'; however, 'G.213' provided a greater quantity of low-size fruits when compared to 'G.210'.

DISCUSSION

In both the cultivars and experimental areas, it was possible to divide the rootstocks into two groups based on vigor. The G.814 and G.210 rootstocks were allocated to the group of rootstocks that induced greater vigor (semi-dwarfs) to the canopy cultivars and the G.202 and G.213 rootstocks formed the group of rootstocks that induced smaller vigor (dwarfs) to the cultivars. The dwarfing rootstocks were ∼38% less vigorous than the semi-dwarfing rootstocks. The classification of 'dwarf rootstock' is related to the efficient control of the vigor of trees induced by rootstock (Denardi et al., 2015). Thus, it is possible to claim that even under extreme replanting conditions the evaluated Geneva rootstocks retained the same vigor characteristics as noted by Denardi et al. (2016) and Macedo et al. (2021) in replanting areas.

In São Joaquim and Vacaria, the 'Gala Select' apple trees in the G.210 rootstock had high accumulated productivity (2 years), and hence these trees were able to express a high productive potential. Both 'G.210' and 'G.814' were categorized as semi-dwarfs; however, the lower vigor of 'G.210' compared to 'G.814' made it more efficient than 'G.814'. Thus, in extreme replanting conditions, greater vigor combined with yield efficiency seems to be related to the productive performance of apple trees. The high accumulated productivity of the G.210 rootstock was also observed by Reig et al. (2020) in an experiment developed in a replanting area in the United States of America (USA), using Super Chief Delicious cultivar.

For the Gala Select cultivar in São Joaquim, the non-fallow condition reduced the productivity of the trees on all rootstocks, especially on 'G.213', which showed 17% less productivity at the end of two harvests when compared to 'G.210' (the most productive). In Vacaria area, due to the lack of thinning in 2019, yields started at a higher level than in the São Joaquim area, resulting in a low increase in production for all rootstocks in the next harvest. The G.213 rootstock was the most affected as it showed less vigor. According to Denardi et al. (2018), on replanting soil conditions, the performance of apple trees may vary according to the rootstock that is used. In general, the most vigorous rootstocks are less affected by apple replant disease when compared to the dwarfing ones, and the tolerance of their root system to harmful agents has a crucial role to play (Isutsa and Merwin, 2000; Leinfelder and Merwin, 2006).

Macedo et al. (2021) evaluated the productive performance of the Gala Select cultivar grafted on Geneva 'G.213' and on rootstocks currently used in southern Brazil (M.9 and Maruba/M.9) in virgin and replanting areas. In the replanting area, fallow period of 12 months was grown before conversion of the orchard. The mean productivity of 'G.213' over a period of 4 years was around 21 $t.ha^{-1}$ in both areas, which was very similar to the value of 22 $t.ha^{-1}$ found in our study (**Table 1**). The cumulative yield in virgin area was higher than in the replanting area over the period of 4 years. However, virgin areas are no longer widely available. Thus, it is important to assess the real need for fallow periods before the orchards are converted. The average and accumulated yields of the replanting area with fallow were numerically smaller than our results obtained for the replanting area without fallow (extreme replanting conditions). Both replanting areas took a year longer than the virgin area to start production. This result shows that trees grafted mainly on dwarfing rootstocks require more time to reach vegetative conditions sufficient for the expected productivity in replanting areas.

However, the 12-month fallow replanting area remained without apple cultivation for 12 months, which is 1 year longer than for the replanted area without fallow. Thus, the productivities obtained from this work in comparison with those published by Macedo et al. (2021) suggests the possibility of immediate conversion of apple orchards of the Gala Select cultivar grafted onto the Geneva G.213 rootstock.

The mean yield efficiency of 'G.213' was around 1.12 in the trials by Macedo et al. (2021), while that obtained from this work was 1.14. In other words, 'G.213' maintained its yield efficiency even under extreme replanting conditions. Thus, it is noteworthy that the main characteristics of these rootstocks, such as yield efficiency and production stability, remain unchanged even under extreme replanting conditions. Macedo et al. (2021) also highlighted that the G.213 rootstock had the lowest alternation bearing in replanting soil, making it a rootstock of high and stable productivity.

The Gala Select cultivar on the G.213 rootstock formed trees with greater yield efficiency than when grafted on 'G.210', in São Joaquim and Vacaria. According to Robinson et al. (2006), some pieces of evidence affirm the direct relationship between yield efficiency and the ananizing effect of rootstocks. The average increment of production per increment of TCSA of 'G.213' was 50.83 tons, while that of 'G.210' was 6.03 tons. In other words, for a small difference in TCSA from 1 year to the next, 'G.213' increased its production by many more tons when compared to 'G.210'. In Vacaria, due to the superiority of yield efficiency (30% higher than 'G.210'), the difference in productivity between 'G.213' and 'G.210' (more productive) was ∼18%. This result corroborates with Rufato et al. (2015) and Macedo et al. (2021), who observed greater yield efficiency among apple trees grafted on G.213 rootstock in replanting areas, based on an experiment developed in Vacaria.

TABLE 1 | Yield of the Gala Select cultivar grafted on 'G.213', in the virgin area, the replanting area with fallow, and the replanting area without fallow in Vacaria.

	Implantation – 2011			Implantation - 2017
Year	**Virgin soil**	**Replanting area (with fallow)**	**Year**	**Extreme replanting area (without fallow)**
2012	–	–	2018	–
2013	3.2	–	2019	-
2014	30.6	26.6	2020	24.88
2015	29.4	16.5	2021	20.46
Mean	**21.07**	**21.55**	**Mean**	**22.67**
Sum	**63.2**	**43.1**	**Sum**	**45.34**
2016	44.6	45.9		
2017	57.5	38.4		
2018	67.7	58.6		
2019	69.2	37.9		
2020	45.2	44.8		
Mean	**43.43**	**38.39**		
Sum	**347.4**	**268.7**		

Results related to virgin area and replanting area with fallow were published by Macedo et al. (2021). Results related to the replanting area without fallow (extreme replanting conditions) were obtained from the present work.

In São Joaquim area, 'Fuji Suprema' grafted on the G.210 rootstock produced trees with high accumulated productivity within 2 years of harvest, followed by the G.814 rootstock. In Vacaria, the Fuji Suprema cultivar in G.814 and G.210 rootstocks produced trees that were equally more productive in the same 2 years. The G.213 rootstock produced trees with lower productivity and greater yield efficiency. However, the yield efficiency of the G.213 rootstock compared to semi-dwarf rootstocks at 'Fuji Suprema' was 39% less than at 'Gala Select'. This relationship explains the lower productivity of the Fuji Suprema cultivar by the G.213 rootstock, because the Fuji Suprema cultivar is more vigorous and is better adapted to more vigorous rootstocks than the Gala Select cultivar (Denardi et al., 2018).

From the results obtained on the accumulated productivity of 2 years, it was possible to infer that the dwarf rootstocks G.202 and G.213 were not able to express all their productive potential for the Gala Select cultivar, under extreme conditions of replanting. The replanting conditions delayed the development of dwarf rootstocks, especially the G.213 rootstock, which required more time to provide the expected productive performance for the Gala Select cultivar. One of the characteristics of dwarf rootstocks is the precocious production. According to Fazio and Robinson (2019), the dwarfing Geneva® rootstocks are much more precocious, with flowering in the first year in an orchard or even in a nursery. However, under extreme conditions of replanting, the expected precocity was not observed. In this condition, 'G.210' produced more than 'G.213' accumulated in the first two crops. However, 'G.213' maintained its characteristic of yield efficiency, with 39% less vigor and 37% more yield efficiency than G.210. As described by Robinson et al. (2011), it is also necessary to consider that greater vigor can result in a laborious orchard.

For the Fuji Suprema cultivar, the effect of the non-fallow condition was less noticeable when comparing the productive performance on the rootstocks analyzed. This is because the Fuji Suprema cultivar already showed higher productivity when grafted on semi-dwarf rootstocks (Pasa et al., 2017). Thus, the more vigorous rootstocks G.210 and G.814 remain important alternatives for the productivity of the Fuji Suprema cultivar, even under extreme replanting conditions.

As for the fruit quality, the G.213 rootstock provided sweeter fruits in both areas and cultivars, indicating the characteristic of possible harvest anticipation. As for the fruit size, the G.213 dwarf rootstock provided a greater quantity of high-size fruits in the Gala Select cultivar and the semi-dwarf G.210 rootstock in the Fuji Suprema cultivar. The fruit size of the Gala Select cultivar provided by G.213 is in accordance with Denardi et al. (2015), who compared G.213 and G.210 rootstocks and concluded that the greatest 'Gala Select' fruit weight was by the G.213 rootstock. However, Pasa et al. (2017) evaluated the fruit size in the Fuji Suprema cultivar for different rootstocks and concluded that the differences in fruit weight are not consistent along the years and are probably not due to rootstock influence.

In general, the fallow time normally respected in the process of reconverting apple orchards has always been considered essential to guarantee the complete development of rootstocks and canopy cultivars. However, this fallow time economically results in the loss of a production crop. In both experimental areas, it was necessary to postpone the first harvest and carry out total thinning to allow the vegetative development of the trees. Thus, despite the immediate conversion, the orchards that did not have a fallow period took the same time to start production as if they had gone through the fallow period. Compared to the virgin area, notwithstanding the overall replant tolerance of Geneva series rootstocks, the non-fallow reduced the productivity by mainly affecting the less vigorous rootstocks that needed about

three crops to overcome the allelopathic effect of the soil and start growing normally. However, on comparing the replanting areas (with and without fallow), it was found that both took the same time to start production after implementation and had the same accumulated productivity in 4 years. However, the one with fallow remained a year without any cultivation. Thus, it can be stated that immediate conversion, without fallowing, can be considered as a strategy in the absence of virgin area for the implantation of new orchards.

Considering the goal of 150 tons in five harvests to cover the costs of orchard implanting, in non-fallow conditions, the G.210 semi-dwarf rootstock was considered to be a good alternative for Gala Select cultivar. Taking into account that the productive potential tends to increase in the next harvest and considering an average of 27.4 tons in the first two harvests, the prospect of reaching the goal was feasible. For the Fuji Suprema cultivar, the G.210 rootstock was also the one that best adapted to the extreme replanting conditions. Productivity was also reduced; however, it resulted in an average of 37.8 tons, ensuring that the goal in five harvests is easily exceeded.

Thus, the immediate conversion leads to the need for 2 years for vegetative formation of the trees and reduces productivity in the first harvest. However, even with reduced productivity in the first harvest, the G.210 rootstock proved to be capable of achieving a productivity of at least 150 tons in five harvests.

CONCLUSIONS

The immediate reconversion of the orchards without fallowing, results in 2 years without vegetative formation for the trees. With or without fallow, the time that elapsed from the orchard implantation until the first commercial harvest remained the same. In replanting area with fallow, the time that elapsed from the orchard eradication until the first commercial harvest was 1 year longer than replanting areas without fallow (extreme replanting conditions).

The non-fallow condition does not alter the difference in vigor and fruit quality between the rootstocks. However, it results in lower productivity, mainly affecting less vigorous rootstocks that need about 3 years to overcome the allelopathic effect of the soil and start growing normally.

The G.210 rootstock is an alternative for the immediate conversion of apple orchards of Gala Select and Fuji Suprema cultivars, in southern Brazil.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LR carried out conceptualization, methodology, validation, supervision, project administration, and funding acquisition. PS was involved in investigation and validation. AK was involved in writing–original draft preparation and resources. AB carried out investigation and data curation. TM contributed to the methodology. JW carried out the investigation. GF contributed to the methodology, as well as the writing, reviewing, and editing of the draft. DP was involved in the writing, reviewing, and editing of the draft, as well as the visualization and formal analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), the National Council for Scientific and Technological Development (CNPq), and the Foundation for Support of Research and Innovation of the State of Santa Catarina (FAPESC).

ACKNOWLEDGMENTS

The authors would like to thank the companies Mareli and Agropecuária Schio Ltda for the availability of orchards for the tests, the institutions CAV-UDESC, Capes, Fapesc, and CNPq, and the companies Agromillora and Catarinense Nursery.

REFERENCES

Afonso, S., Ribeiro, C., Bacelar, E., Ferreira, H., Oliveira, I., Silva, A. P., et al. (2017). Influence of training system on physiological performance, biochemical composition and antioxidant parameters in apple tree (Malus domestica Borkh.). *Sci. Horticult.* 225, 394–398. doi: 10.1016/j.scienta.2017.07.037

Beers, E. H., Cockfield, S., and Fazio, G. (2006). "Biology and management of woolly appleaphid, Eriosoma lanigerum (Hausmann)," in *Proceedings of the IOBC University of Lleida* (Lleida), 4–6.

Denardi, F., Kvitschal, M. V., Basso, C., Boneti, J. I. D. S., and Katsurayama, Y. (2015). Agronomic performance of the Geneva® serie apple rootstocks in the southern of Brazil. *Rev. Bras. Fruticult.* 37, 104–111. doi: 10.1590/0100-2945-438/14

Denardi, F., Kvitschal, M. V., Basso, C., Boneti, J. I. S., and Katsurayama, Y. (2016). Performance of new apple rootstocks for Gala variety in Southern Brazil. *Crop Breed. Appl. Biotechnol.* 16, 147–152. doi: 10.1590/1984-70332016v16n2n22

Denardi, F., Kvitschal, M. V., and Hawerroth, M. C. (2018). Yield performance of apple rootstocks of the Geneva series on replanting soil. *Pesquisa Agro. Bras.* 53, 924–933. doi: 10.1590/s0100-204x2018000800007

Embrapa (2004). *Empresa Brasileira de Pesquisa Agropecuária. Manual de Segurança e Qualidade para a Cultura da Maçã*. Brasilia: EMBRAPA/SEDE.

Fazio, G., Aldwinckle, H., and Robinson, T. (2013). Unique characteristics of Geneva® apple rootstocks. *New York Fruit Q.* 21, 25–28.

Fazio, G., Kviklys, D., Grusak, M. A., and Robinson, T. (2012). Soil pH, soil type and replant disease affect growth and nutrient absorption in apple rootstocks. *New York Fruit Q.* 20, 22–28.

Fazio, G., and Robinson, T. (2019). *Advances in the Development and Utilization of Fruit Tree Rootstocks: A Case Study for Apple.* London: Burleigh Dodds Science Publishing Limited.

Gjamovski, V., and Kiprijanovski, M. (2011). Influence of nine dwarfing apple rootstocks on vigour and productivity of apple cultivar 'Granny Smith'. *Sci. Horticult.* 129, 742–746. doi: 10.1016/j.scienta.2011.05.032

Harrison, N., Harrison, R. J., Barber-Perez, N., Cascant-Lopez, E., Cobo-Medina, M., Lipska, M., et al. (2016). A new three-locus model for rootstock-induced

dwarfing in apple revealed by genetic mapping of root bark percentage. *J. Exp. Bot.* 67, 1871–1881. doi: 10.1093/jxb/erw001

Isutsa, D. K., and Merwin, I. A. (2000). Malus germplasm varies in resistance or tolerance to apple replant disease in a mixture of New York orchard soils. *HortScience* 35, 262–268. doi: 10.21273/HORTSCI.35.2.262

Köppen, W. (1948). *Climatologia: Con un Estudio de los Climas de la Terra*. México. Fondo Cult. Econ.

Kviklys, D., Liaudanskas, M., Janulis, V., Viškelis, P., Rubinskiene, M., Lanauskas, J., et al. (2015). Rootstock genotype determines phenol content in apple fruits. *Plant Soil Environ.* 60, 234–240. doi: 10.17221/71/2014-PSE

Kviklys, D., Robinson, T. L., and Fazio, G. (2016). "Apple rootstock evaluation for apple replant disease," in *XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014)* (Brisbane, QLD), 425–430.

Leinfelder, M. M., and Merwin, I. A. (2006). Management strategies for apple replant disease. *New York Fruit Q.* 14, 39–42.

Macedo, T. A., Silva, P. S., Sander, G. F., Rossi, A., Kretzschmar, A. A., Petry, D., et al. (2021). G.213 rootstock – Alternative to apple tree cultivation in different planting areas in southern Brazil. *Sci. Horticult.* 286:110219. doi: 10.1016/j.scienta.2021.110219

Macedo, T. A., Silva, P. S., Sander, G. F., Welter, J. F., Rufato, L., and Rossi, A. (2019). Productivity and quality of 'Fuji Suprema' apple fruit in different rootstocks and growing conditions. *Sci. Horticult.* 256:108651. doi: 10.1016/j.scienta.2019.108651

Pasa, M. D. S., Brighenti, A. F., Katsurayama, J. M., Silva, C. P., Ciotta, M. N., and Carra, B. (2017). Productive performance of 'fuji' apple trees on 'JM' series rootstocks, in São Joaquim/SC. *Rev. Bras. Fruticult.* 39:e-090. doi: 10.1590/0100-29452017090

Pasa, M. D. S., Katsurayama, J. M., Brighenti, A. F., Araújo Filho, J. V. D., and Boneti, J. I. D. S. (2016). Performance of 'Imperial Gala' and 'Mishima Fuji' apples on different rootstocks. *Pesquisa Agropecuária Bras.* 51, 17–26. doi: 10.1590/S0100-204X2016000100003

Pereira, T. P., Fontana, D. C., and Bergamaschi, H. (2009). The climate of the Campos de Cima da Serra Region, Rio Grande do Sul sate, Brazil: thermal and water conditions. *Pesquisa Agropecuária Gaúcha* 15, 145–157.

Reig, G., Lordan, J., Hoying, S., Fargione, M., Donahue, D. J., Francescatto, P., et al. (2020). Long-term performance of 'Delicious' apple trees grafted on Geneva® rootstocks and trained to four high-density systems under New York State climatic conditions. *HortScience* 55, 1538–1515. doi: 10.21273/HORTSCI14904-20

Robinson, T. L., Fazio, G., Aldwinckle, H. S., Hoying, S. A., and Russo, N. (2006). Field performance of Geneva® apple rootstocks in the Eastern USA. *Sodininkyste ir darŽininkyste* 25, 181–191.

Robinson, T. L., Hoying, S. A., and Fazio, G. (2011). Performance of Gevena rootstocks in on-farm trials in New York. *Acta Horticult.* 903, 249–256. doi: 10.17660/ActaHortic.2011.903.31

Rufato, L., Rossi, A., Macedo, T., and Peter, F. (2015). *Resultados de Pesquisa de Porta-Enxertos da Série G de Geneva e Perspectivas Futuras. Embrapa Uva e Vinho*. Bento Gonçalves: INFOTECA-E. Available online at: http://www.infoteca.cnptia.embrapa.br/infoteca/handle/doc/1076047

Tworkoski, T., and Fazio, G. (2016). Hormone and growth interactions of scions and size-controlling rootstocks of young apple trees. *Plant Growth Regul.* 78, 105–119. doi: 10.1007/s10725-015-0078-2

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 October 2021
doi: 10.3389/fpls.2021.726461

Relationship Between the Xylem Anatomy of Grapevine Rootstocks and Their Susceptibility to *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*

Charis K. Ramsing[1], David Gramaje[2], Sara Mocholí[1], Javier Agustí[3], Félix Cabello Sáenz de Santa María[4], Josep Armengol[1] and Mónica Berbegal[1]**

[1] Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Valencia, Spain, [2] Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas, Universidad de la Rioja–Gobierno de La Rioja, Logroño, Spain, [3] Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas (CSIC) Universitat Politècnica de València, Valencia, Spain, [4] Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA), Madrid, Spain

OPEN ACCESS

Edited by:
Vittorino Novello,
University of Turin, Italy

Reviewed by:
Alban Jacques,
Purpan Engineering School, France
Ales Eichmeier,
Mendel University in Brno, Czechia

***Correspondence:**
Mónica Berbegal
mobermar@etsia.upv.es
David Gramaje
david.gramaje@icvv.es

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

Received: 16 June 2021
Accepted: 06 September 2021
Published: 12 October 2021

Citation:
Ramsing CK, Gramaje D, Mocholí S, Agustí J, Cabello Sáenz de Santa María F, Armengol J and Berbegal M (2021) Relationship Between the Xylem Anatomy of Grapevine Rootstocks and Their Susceptibility to Phaeoacremonium minimum and Phaeomoniella chlamydospora. Front. Plant Sci. 12:726461. doi: 10.3389/fpls.2021.726461

Fungal grapevine trunk diseases (GTDs) are some of the most pressing threats to grape production worldwide. While these diseases are associated with several fungal pathogens, *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* are important contributors to esca and Petri diseases. Recent research has linked grapevine xylem diameter with tolerance to *Pa. chlamydospora* in commercial rootstocks. In this study, we screen over 25 rootstocks for xylem characteristics and tolerance to both *Pa. chlamydospora* and *Pm. minimum.* Tolerance was measured by fungal incidence and DNA concentration (quantified via qPCR), while histological analyses were used to measure xylem characteristics, including xylem vessels diameter, density, and the proportion of the stem surface area covered by xylem vessels. Rootstocks were grouped into different classes based on xylem characteristics to assess the potential association between vasculature traits and pathogen tolerance. Our results revealed significant differences in all the analyzed xylem traits, and also in DNA concentration for both pathogens among the tested rootstocks. They corroborate the link between xylem vessels diameter and tolerance to *Pa. chlamydospora.* In *Pm. minimum,* the rootstocks with the widest xylem diameter proved the most susceptible. This relationship between vasculature development and pathogen tolerance has the potential to inform both cultivar choice and future rootstock breeding to reduce the detrimental impact of GTDs worldwide.

Keywords: tolerance, vascular pathogens, Esca, Petri, fungal trunk diseases

INTRODUCTION

Esca and Petri diseases are two major fungal grapevine trunk diseases (GTDs) that currently and significantly threaten grapevine production (Gramaje et al., 2018). These diseases are present in every grape-growing region worldwide. The complex etiology of such diseases comprises many fungal species. These include the pathogen *Phaemoniella chlamydospora* and several

Phaeoacremonium species with *Phaeoacremonium minimum* being the most widely distributed and the most commonly isolated species of this genus affecting grapevine (Bertsch et al., 2013; Gramaje et al., 2015, 2018).

Esca symptoms can emerge in either mild or severe forms. Severe esca is also called "apoplexy" and can involve abrupt wilting and even grapevine death. Milder or chronic forms usually feature deteriorating foliage. This begins with interveinal chlorosis that progresses to necrotic tissue and results in "tiger-striped" leaves. Berries may display scattered spots known as "black measles." The internal wood symptoms of affected plants include white soft-rot surrounded by a dark line and cinnamon to black spots (Bertsch et al., 2013; Gramaje et al., 2018).

Petri disease more frequently affects grafted vines in grapevine nurseries and young vines in newly planted vineyards and causes plant stunting and dieback (Gramaje and Armengol, 2011; Gramaje et al., 2018). Additional external symptoms include delayed bud break, weak vegetative growth, and occasional interveinal chlorosis that leads to necrosis and leaf wilt. Internal symptoms are often characterized by dark discoloration on the grapevine trunk and cordons, and by brown to black vascular streaking (Mostert et al., 2006b; Gramaje et al., 2010, 2018).

Esca and Petri diseases have become increasingly prevalent in recent years due to several factors. Specifically, (i) the worldwide grapevine planting boom in the 1990s, (ii) changes in cultural practices that favor fungal infections, and (iii) lack of effective chemicals preventing fungal infections in both nurseries and vineyards (Gramaje et al., 2018; Mondello et al., 2018). Unfortunately, there are no current viable or universal methods to manage these diseases, thus contributing to their widespread across all vine-growing areas (Mondello et al., 2018).

Host resistance is almost always the ideal management method for plant pathogens, especially in perennial crops like grapevine, where re-establishing fields year after year makes production costly (Gramaje et al., 2018). The estimated worldwide annual financial cost of replacing plants that have died of GTDs is more than € 1,132 billion (US$ 1.502 billion) (Hofstetter et al., 2012). However, while the susceptibility of rootstocks and cultivars differ to some GTDs, no cultivar or species in *Vitis* has been found to express complete resistance (Eskalen et al., 2001; Feliciano et al., 2004; Gramaje et al., 2010; Martínez-Diz et al., 2019). Instead, all grapevine rootstocks and cultivars can be potentially infected by GTD fungi, but symptom expression and severity vary among cultivars. Quantitative resistance does not prevent infection, but limits crop losses and damage and reduce the epidemic over time (Suthman et al., 2007; Poland et al., 2008; St. Clair, 2010). Quantitative resistance is typically not strain-specific and thus, tends to be effective across all the strains of a pathogen population (Niks et al., 2015).

A better understanding of disease development is essential to identify novel genetic resources for resistance against Esca and Petri diseases. Screening studies to evaluate rootstock susceptibility to *Pa. chlamydospora* and *Pm. minimum* have been conducted by artificial inoculation under field (Gramaje et al., 2010), under controlled conditions using *in vitro* grapevine shoots (Zanzotto et al., 2008), and greenhouse cuttings (Eskalen et al., 2001). Although these studies were useful, they yielded inconsistent results and did not offer any information about pathogen colonization or rootstock characteristics that might be associated with different susceptibility levels. Identifying physiological characteristics linked with tolerant rootstocks would greatly help breeding toward disease tolerance (Nelson et al., 2018).

The physiological and histochemical alterations associated with *Pa. chlamydospora* infections are subjects of more recent interest. Studies employing green fluorescent protein (GFP)-transformed *Pa. chlamydospora* and *Pm. minimum* have demonstrated that the fungi are found mainly in woody tissue and concentrates around occluded xylem vessels (Landi et al., 2012; Pierron et al., 2015). *Pa. chlamydospora* is known to induce the host plant to produce tyloses containing various tannins and phenolic compounds, which may explain these obstructions (Lorena et al., 2001). Similar occlusion structures are found in many fungal wilt diseases and are considered to plant defense mechanisms that seal off and trap pathogens in an enclosed space where defense compounds accumulate (Fradin and Thomma, 2006). These findings indicate that the plant vasculature may play a more relevant role in disease development than expected. Indeed, one key determinant of plant resistance to vascular infections lies in the ability of the host to successfully compartmentalize invaders at the xylem level (Pouzoulet et al., 2020a). More specifically, the impact of xylem vessel diameter on compartmentalization efficiency and, thus, on vascular pathogen movement has been analyzed for *Pa. chlamydospora* in grapevine (Pouzoulet et al., 2017, 2020a). Recent studies into grapevine xylem anatomy have shown that grapevine rootstocks display varying xylem vessel diameters, and the density of xylem vessels with diameters above 120 μm correlates with the *Pa. chlamydospora* DNA concentration. These observations support the hypothesis that a wider xylem vessel would be harder to obstruct (Pouzoulet et al., 2014, 2017). Comparable results have also been found in Dutch elm disease caused by the vascular pathogen *Ophiostoma novo-ulmi* (Solla and Gil, 2002; Venturas et al., 2014).

There is a need to provide further insights into the idea that vasculature anatomy is a driver in grapevine tolerance to esca and Petri diseases. In our study, shoots from grapevine rootstocks representing different agronomical characteristics and *Vitis* spp. crosses were inoculated with *Pa. chlamydospora* and *Pm. minimum* to determine their susceptibility to these pathogens using fungal isolation and DNA concentration (the latter determined by qPCR) as the main criteria. Xylem anatomy parameters such as xylem density, xylem vessels surface area, and vessel diameter were also measured. The specific objectives of this study were to (i) evaluate differences in pathogen tolerance to *Pa. chlamydospora* and *Pm. minimum* and in xylem vessel characteristics among the studied rootstocks, and (ii) investigate the relationship between tolerance to fungal pathogens and xylem anatomy.

MATERIALS AND METHODS

Grapevine Rootstock Inoculation

Shoots from different grapevine rootstocks were selected to represent different agronomical characteristics and *Vitis* spp. crosses (**Table 1**). Referenced well-characterized isolates of *Pa.*

TABLE 1 | List of the rootstocks used in the study.

Rootstock	Parents	Origin[a]	*Phaeomoniella chlamydospora* inoculation[b]	*Phaeomoniella chlamydospora* qPCR	*Phaeoacremonium minimum* inoculation	*Phaeoacremonium minimum* qPCR	Histological analysis
Berlandieri resseguier 1	*V. berlandieri*	El Encín	*	*	*	*	*
Blanchard 1	*V. berlandieri* × *V. vinifera*	El Encín	*	*	*	*	*
Cardeden 31		El Encín	*		*		
Castel 14539	(*V. vinifera* Chasselas × *V. rupestris*) × *V. vinifera* Chasselas	El Encín	*		*		
Castel 196–17	(*V. vinifera* × *V. rupestris*) × *V. riparia*	El Encín	*	*	*	*	*
Castel 6736	*V. riparia* × *V. rupestris*	El Encín	*	*	*	*	*
Castel 6971	*V. riparia* × *V. rupestris*	El Encín	*	*	*	*	*
Castel 7605	*V. riparia* × *V. berlandieri*	El Encín	*	*	*	*	*
Couderc 1202	*V. vinifera* × *V. rupestris*	El Encín	*	*	*	*	*
Couderc 161–49	*V. riparia* × *V. berlandieri*	Viveros Villanueva	*	*			*
COUDERC 1616	*V. longii* × *V. riparia*	El Encín	*		*	*	*
Couderc 3309	*V. riparia* × *V. rupestris*	El Encín	*		*		
Couderc 404	*V. vinifera* × *V. rupestris*	El Encín	*		*		
Couderc 601	Bourrisqouou × *V. rupestris*	El Encín	*		*		
Couderc 9	(*V. riparia* × *V. rupestris*) × *V. vinifera*	El Encín	*		*		
Escuela montpellier 333	*V. vinifera* × *V. berlandieri*	El Encín	*	*	*	*	*
Evex jerez 13–5	*V. berlandieri*	El Encín	*	*	*	*	*
Fercal	*V. berlandieri* × (*V. berlandieri* × Novo Mexicana)	El Encín	*	*	*	*	*
Foex 34-E	*V. berlandieri* × *V. riparia*	El Encín	*		*		
Grezot G1	(*V. longii* × *V. riparia*) × *V. rupestris*	El Encín	*	*	*	*	*
Grimaldi 791	*V. vinifera* × (*V. riparia* × *V. rupestris*)	El Encín	*		*	*	*
Malague 44–53		El Encín	*		*		
Martinez zaporta 5A	*V. vinifera* Chasselas × *V. berlandieri*	El Encín	*		*		
Millardet grasset 41B	*V. vinifera* × *V. berlandieri*	El Encín	*	*	*	*	*
Millardet 145	*V. vinifera* × (Cordifolia-Rupestris Grasset)	El Encín	*		*		
Millardet 33A-1	*V. vinifera* × *V. rupestris*	El Encín	*		*	*	*
MILLARDET 453	Aramon × Millardet	El Encín	*		*		
Millardet grasset 19–62	*V. vinifera* × *V. berlandieri*	El Encín	*		*		
Millardet grasset 420A	*V. berlandieri* × *V. riparia*	El Encín	*	*	*		*
Oberlin 595	*V. riparia* × *V. vinifera*	El Encín	*		*		
Paulsen 1103	*V. berlandieri* × *V. rupestris*	El Encín	*		*		
Paulsen 1447	*V. berlandieri* × *V. rupestris*	El Encín	*		*		
Ramsey		INRA Montpellier	*				
RG1		Vitis Navarra	*		*		
RG10		Vitis Navarra	*		*		
RG2		Vitis Navarra	*		*		
RG3		Vitis Navarra	*		*		

(Continued)

TABLE 1 | Continued

Rootstock	Parents	Origin[a]	*Phaeomoniella chlamydospora* inoculation[b]	*Phaeomoniella chlamydospora* qPCR	*Phaeoacremonium minimum* inoculation	*Phaeoacremonium minimum* qPCR	Histological analysis
RG4		Vitis Navarra	*		*		*
RG6		Vitis Navarra	*		*		
RG7		Vitis Navarra	*		*		
RG8		Vitis Navarra	*		*		
RG9		Vitis Navarra	*		*	*	*
Richter 99	*V. berlandieri* × *V. rupestris*	El Encín	*		*		
Richter 110	*V. berlandieri* × *V. rupestris*	El Encín	*	*			*
Richter 31	*V. berlandieri* × Novo Mexicana	El Encín	*	*	*	*	*
Riparia grand glabre	*V. riparia*	El Encín	*	*	*	*	*
Ruggeri 131	*V. berlandieri* × *V. rupestris*	El Encín	*		*		
Ruggeri 140	*V. berlandieri* × *V. rupestris*	El Encín	*	*	*	*	*
Ruggeri 267	*V. berlandieri* × *V. riparia*	El Encín	*	*	*	*	*
Ruggeri 343	*V. berlandieri* × *V. riparia*	El Encín	*	*	*	*	*
Rupestris du lot	*V. rupestris*	El Encín	*		*		
Rupestris fort worth 1	*V. rupestris*	El Encín	*	*	*	*	*
Seibel 397	Herbemot touzan × Sauvignon	El Encín	*		*		
SO4	*V. berlandieri* × *V. riparia*	El Encín	*	*	*	*	*
Teleki 10A	*V. berlandieri* × *V. rupestris*	El Encín	*		*		
Teleki-Kober 5BB	*V. berlandieri* × *V. riparia*	El Encín	*	*			*

[a] *El Encín, Alcalá de Henares (Madrid, Spain); Viveros Villanueva, Larraga (Navarra, Spain); Vitis Navarra, Larraga (Navarra, Spain).*
[b] *Analysis performed = *.*

chlamydospora (Pch-184, obtained in 2004 at Sinarcas, Valencia, from Tempranillo variety grafted onto 110 R rootstock) and *Pm. minimum* (Pal-45, obtained in 2002 at Argamasilla de Alba, Ciudad Real, from Tempranillo variety grafted onto Richter 110 rootstocks) were used for inoculation. These isolates were maintained in 15% glycerol solution at −80°C into 1.5 ml cryovials at the fungal collection of the Instituto Agroforestal Mediterráneo—Universitat Politècnica de València (IAM-UPV) (Spain).

Rootstock shoots were cut into smaller cutting fragments (10 cm length) with at least one terminal node. The base of these fragments was immersed for 30 min in *Pa. chlamydospora* and *Pm. minimum* spore suspensions (10^6 conidia/ml) obtained from the above-mentioned isolates based on the methodology described by Gramaje et al. (2010). Nine cuttings per cultivar and fungal species were inoculated and nine cuttings were immersed in sterile distilled water as negative controls.

The inoculated cuttings were randomly distributed in a hydroponic culture system as described by Sosnowski et al. (2016). These cuttings were placed inside holes made in 2 cm-thick polystyrene boards to ensure that the base of cuttings dropped ∼1 cm below the boards. These boards were floated on water dosed with a soluble fertilizer (25% Hoagland solution) in plastic tubs inside a plant growth chamber. The liquid substrate received a continuous oxygen supply by an aquarium air pump. Cuttings were maintained at ∼25°C for 45 days. The experiment was repeated once. The extent of fungal colonization on shoots was evaluated 45 days after inoculation by pathogen isolation and quantifying the amount of fungal DNA by a qPCR assay.

Fungal Isolation

Cuttings were collected 45 days after inoculation. The bark was removed using a sharp knife and the exposed wood was surface disinfected for 1 min in 1.5% sodium hypochlorite solution before being rinsed twice in sterile distilled water. For each cutting, wood discs (4 mm) were cut using sterile secateurs at a distance of 4 cm above the inoculated base to assess the presence of *Pa. chlamydospora* or *Pm. minimum*. One-half of each disc was used for pathogen isolation and the other half for DNA extraction (**Table 1**).

For fungal isolation purposes, six small segments were plated on potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g/L of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated at 25°C in the dark for 7 to 10 days, and all the colonies were transferred to PDA. A cutting was considered infected when at least one positive isolation point was obtained for the inoculated pathogens. Identification of *Pa. chlamydospora* or *Pm. minimum* was based on the colony morphology (Crous and Gams, 2000; Mostert et al., 2006a). By considering these results, the disease incidence percentage was estimated as the number of infected cuttings of all the total inoculated. Of all the non-inoculated controls, one additional wood disk

from each rootstock cutting was taken and kept for further histological analyses.

DNA Extraction and Quantification

Bark and pith were removed from wood sections with a sterile scalpel before genomic DNA (gDNA) was obtained. A total initial wood mass of 50 mg was analyzed. Each sample was homogenized using a mortar and pestle and placed in 2 ml tubes containing 2 and 4, 3- and 2.35-mm-diameter, respectively, tungsten carbide beads (Qiagen, Hilden, Germany), and 500 µl of cetyl trimethylammonium bromide (CTAB) extraction buffer [2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, and 1% polyvinylpyrrolidone (PVP)]. Tubes were placed in a FastPrep® (MP Biomedicals, Santa Ana, CA, USA) at 124 Hz for 30 s. Subsequently, the DNA extraction procedure was conducted as described by Saito et al. (2013).

For pathogen DNA quantification, the total DNA of the inoculated isolates grown in PDA was extracted with the EZNA Plant Miniprep kit (Omega Bio-Tek, Norcross, GA, USA). Before DNA extraction, the sample was homogenized in 2 ml tubes following the same procedure described before, but with 600 µl of P1 buffer (provided in the kit) instead of CTAB buffer. The concentration (ng/µl) of the obtained gDNA was quantified with the Qubit Fluorometric Quantitation kit (Life Technologies, Carlsbad, CA, USA), which resulted in 18.2 and 5.5 ng/µl for *Pa. chlamydospora* and *Pm. minimum*, respectively. Seven 1:10-fold serial dilutions of gDNA were prepared and used as standards.

Quantitative polymerase chain reaction assays were performed in a final 25 µl volume, and the reaction mixtures contained 12.5 µl of TB Green™ Premix Ex Taq™ (2x) (Tli RNaseH Plus; Takara Bio Inc., Shiga, Japan) and 2 µl of template DNA. The primer sets PchQF/R and PalQF/R described by Pouzoulet et al. (2013) to detect *Pa. chlamydospora* and *Pm. minimum*, respectively, were used at a final concentration of 0.5 µM. Experiments were conducted in a Rotor-Gene Q 5plex HRM instrument (Qiagen), and the reaction conditions were initial denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, and 62°C for 45 s. Additional melting analysis from 50 to 99°C was performed to confirm correct product amplification.

The quantification cycle (Cq) value for each standard gDNA sample was calculated by the Rotor-Gene Q Series software (version 2.3.1) to generate a standard curve for the quantification of *Pa. chlamydospora* and *Pm. minimum,* and to estimate the limit of detection. Both standards and samples were analyzed using four technical replicates and the nomenclature for interpreting all the qPCR results followed the MIQE guidelines, as described by Bustin et al. (2009).

Histological Analysis

For a selected number of rootstocks (**Table 1**), four 3–5 mm-stem fragments were collected and vacuum-infiltrated by fixation for 15 min in 70% EtOH. Samples were then processed for dehydration, eosin staining, and paraffin infiltration using a Leica TP1020 tissue processor (Leica Biosystems, Buffalo Grove, IL, USA). Subsequently, paraffin blocks containing the eosin-stained samples were made and sectioned using a Leica microtom (Leica Biosystems). In all cases, 60 µm sections were produced, floated in 40°C water, and mounted on slides, which were left overnight on a 40°C heated plate. Samples were then de-parafinned through two histoclear baths. Histoclear was removed with 100% ethanol and coverslips were placed on samples and fixed with Merck-Glass fixing media (Merck, Kenilworth, NJ, USA). For the quantitative analyses, photographs were taken for each sample using a Leica DM5000 microscope (Leica Biosystems). For each sample, the diameter and area of at least 100 vessels were measured in both lateral and dorsal interfascicular regions using the Fiji free software. Xylem vessel area occupancy, namely, the xylem vessel surface, was calculated for each analyzed interfascicular region as the total vessel area/total interfascicular region area ratio. The mean xylem vessel diameter (µm), xylem vessel density (no. of vessels/mm^2), and xylem vessel surface (mm^2 xylem/total mm^2 surface area) values were obtained for each rootstock.

Data Analysis

Statistical analyses were run within each variable, both independent and dependent, to test if there were significant differences between rootstocks. Data distribution was right-skewed and justified by running a Kruskal-Wallis test for all the variables except xylem density. Dunn's and Wilcoxon rank sum tests were run for the *post-hoc* analyses. Xylem density was assessed by a one-way ANOVA followed by Tukey's *post-hoc* HSD test.

The imbalance between the number of replicates per sample used for the independent and dependent variables made a direct comparison between both variables difficult. Whereas four replicates per rootstock were used for the histological data (independent variables), from four to sixteen replicates per rootstock were employed for the DNA concentration (dependent variable). To mitigate this issue, the independent variables were sorted into classes to convert them from quantitative variables into qualitative factors based on Pouzoulet et al. (2017). Five classes were established for each variable (diameter, density, surface) and assigned as follows: five diameter classes (<44, 45–54, 55–64, 65–74, ≥75 µm), five density classes [<40, 40–49, 50–59, 60–69, ≥70 (no. vessels/mm^2)], and five surface classes [0.10–0.14, 0.15–0.19, 0.20–0.24, 0.25–0.30, ≥0.31 (mm^2 xylem/total mm^2)]. Kruskal-Wallis tests were run for the histological variable DNA concentration combination, followed by Dunn's and the Wilcoxon ranked sum *post-hoc* tests. To see if there were any differences in incidence and DNA concentration and if histological factors were common between crosses, rootstock varieties were sorted into their parent crosses and the same statistical tests were run. The Kendall rank correlation test was used to investigate the potential correlation between histological traits.

All the data analyses were run in RStudio 1.3.959 with R 4.0.0 (R Core Team, 2020). Essential packages included "agricolae," "ARTool," "dplyr," "FSA," "ggplot2," "ggpubr," and "grDevices" (Wickham, 2016; de Mendiburu, 2019; Kassambara, 2020; Kay and Wobbrock, 2020; Wickham et al., 2020; Ogle et al., 2021).

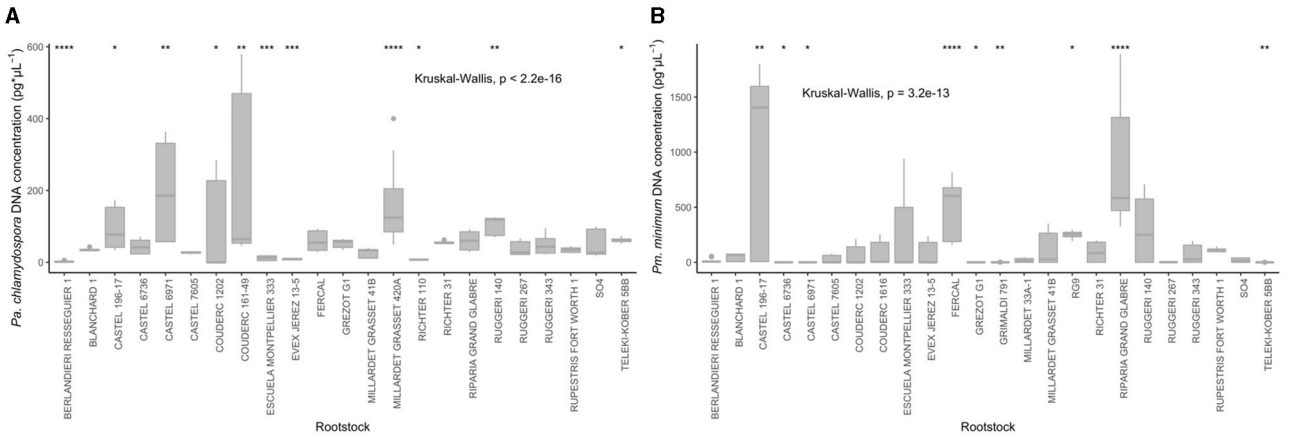

FIGURE 1 | Box plots of the fungal DNA concentration found in differing grape rootstock varieties. **(A)** *Phaeomoniella chlamydospora* DNA concentration in pg/µl considering an initial wood mass for the analysis of 50 mg. **(B)** *Phaeoacremonium minimum* DNA concentration in pg/µl considering an initial wood mass for the analysis of 50 mg. Significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$) determined by the Wilcoxon rank sum test.

RESULTS

Pathogen Tolerance

No significant rootstock effect was observed on either *Pa. chlamydospora* or *Pm. minimum* incidence based on the pathogen isolation from the inoculated cuttings ($P = 0.1451$ and $P = 0.4739$, respectively). All the rootstocks were infected, except Castel 6971 cuttings inoculated with *Pm. minimum*, which had no positive point of isolation. Incidence results vastly varied, with percentage value ranges of 5.5–86.7 and 0–88.9% for *Pa. chlamydospora* and *Pm. minimum*, respectively. Very low *Pa. chlamydospora* incidence levels (<17%) were observed for rootstocks Cardeden 31, Castel 7605, Couderc 601, Paulsen 1447, and Richter 99. Low *Pm. minimum* incidence levels (<17%) were obtained for rootstocks Couderc (9, 1606, and 3309) and Teleki 10A. Incidence levels below 17% were found for Couderc 404, Grimaldi 791, RG10, and Richter 31 rootstock cuttings inoculated with both pathogens (**Supplementary Figure 1**).

The analysis showed significant differences between rootstocks for both pathogens' DNA concentration detected in the inoculated cuttings ($P < 0.0001$, **Figure 1**). The average DNA concentration values ranged from 2.4 to 163.2 pg/µl and from 0.04 to 916.5 pg/µl for *Pa. chlamydospora* and *Pm. minimum*, respectively. Significantly lower *Pa. chlamydospora* DNA concentrations (<15 pg/µl) were detected in rootstocks Berlandieri Resseguier 1, Escuela Montpellier 333, Evex Jerez 13-5, and Richter 110 ($P < 0.0001$, $P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively) while significantly higher *Pa. chlamydospora* DNA concentrations (>160 pg/µl) were detected in Millardet Grasset 420A, Couderc 161–49, and Castel 6971 ($P < 0.0001$, $P < 0.001$, $P < 0.001$, respectively) (**Figure 1A**, **Supplementary Table 1**). For *Pm. minimum*, significantly lower DNA concentrations (<1 pg/µl, $P < 0.05$) were detected in rootstocks Castel 6736 and 6971, Grezot G1, Grimaldi 791, and Teleki Kober 5BB, while significantly higher concentrations (<400 pg/µl) were recorded in rootstocks Castel 196-17, Fercal, and Riparia Grand Glabre ($P < 0.01$, $P < 0.0001$, and $P < 0.0001$, respectively) (**Figure 1B**, **Supplementary Table 1**).

Xylem Anatomy

Apart from the visual differences among xylem vessel characteristics (**Figure 2**), a significant rootstock effect was observed for all the xylem anatomy parameters, including vessels diameter, xylem vessel density, and surface area ($P < 0.01$). The average xylem vessel diameter values ranged from 41.1 to 83.6 µm, with RG4, RG9, and Richter 110 showing significantly wider xylem vessels (<75 µm, $P < 0.01$, $P < 0.05$, and $P < 0.01$ respectively) (**Figure 3A**, **Supplementary Table 2**). The average xylem vessel density values ranged from 31.1 to 110.5 vessels/mm^2 with Blanchard 1 having significantly dense vessels (110.5 vessels/mm^2, $P < 0.0001$; **Figure 3B**, **Supplementary Table 2**). The average xylem vessel surface values ranged from 0.1 to 0.4 mm^2 xylem/total mm^2 surface area with RG4, RG9, Richter 110, Ruggeri 267, and SO4 obtaining significantly higher surface values (<0.245 mm^2 xylem/total mm^2 surface area, $P < 0.05$; **Figure 3C**, **Supplementary Table 2**). Interestingly, rootstocks RG4, RG9, and Richter 110 had significantly different values for each histological parameter and significantly wider vessel diameters (<75 µm, $P < 0.05$), significantly lower xylem densities (<41 vessels/mm^2, $P < 0.05$) and significantly high xylem surface values (<0.245 mm^2 xylem/total mm^2 surface area, $P < 0.05$; **Figures 3A–C**, **Supplementary Table 2**).

A significant correlation was found between vessels diameter and xylem density ($R = -0.59$, $P < 0.01$) and between vessel diameter and xylem surface ($R = 0.35$, $P < 0.01$; **Figures 4A,C**). In contrast, no significant correlation was observed between xylem density and surface ($R = -0.069$, $P = 0.28$; **Figure 4B**).

When comparing rootstock parent crosses rather than individual varieties, all the histological traits were statistically significant. *Vitis berlandieri* × *V. vinifera* showed a significantly

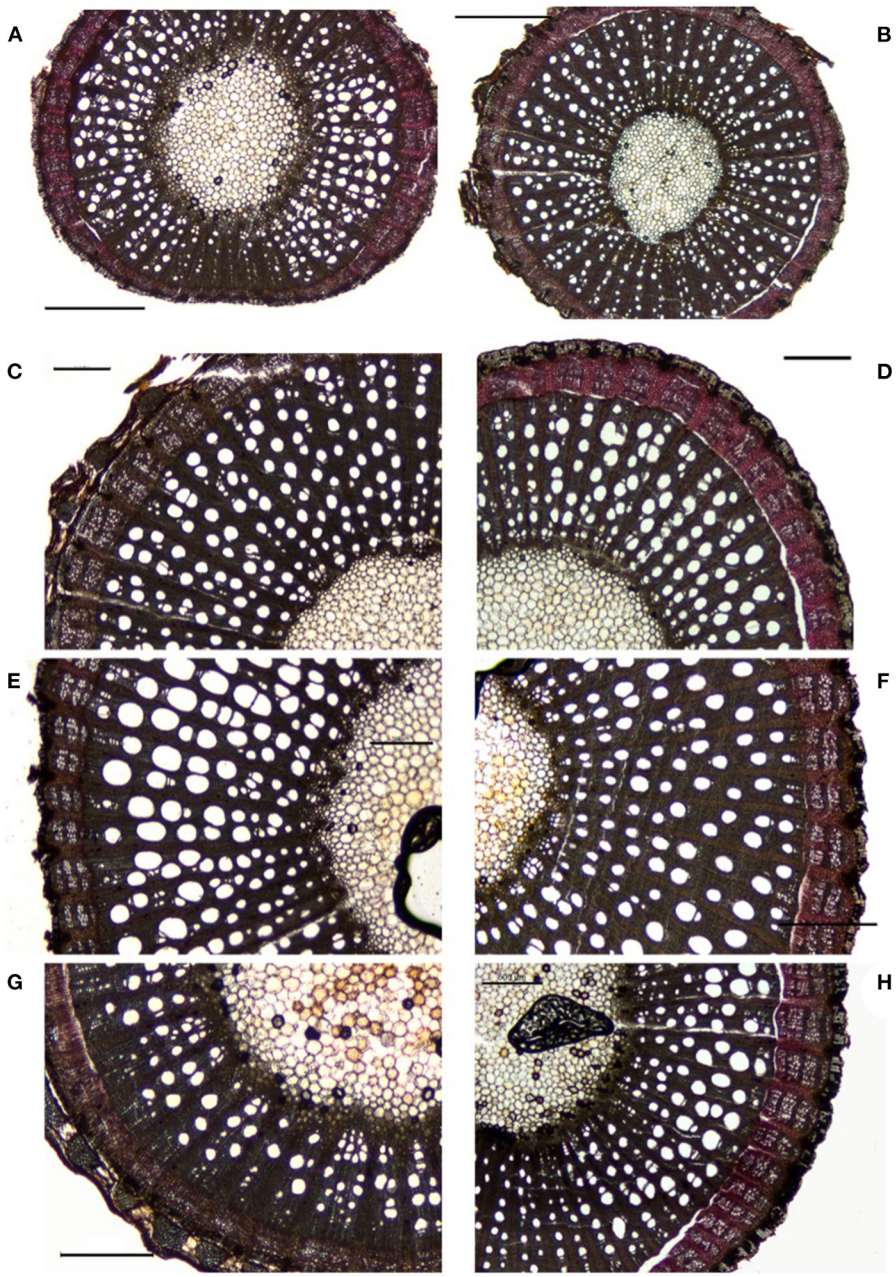

FIGURE 2 | Image analysis for the different rootstocks 60 μm sections. Blanchard 1 **(A)**, Castel 6736 **(B)**, Grezot G1 **(C)**, Rupestris Fort Worth 1 **(D)**, RG9 **(E)**, Richter 110 **(F)**, Grimaldi 791 **(G)**, and SO4 **(H)**. Horizontal bars = 500 μm.

narrower vessel diameter ($P < 0.01$) and significantly higher vessel density ($P < 0.01$), but did not statistically differ for vessel surface (**Figures 5A–C**, **Supplementary Table 3**, **Supplementary Figure 3**). *Vitis berlandieri* × Novo Mexicana, *V. riparia*, *V. riparia* × *V. berlandieri,* and *V. rupestris* also showed statistically different vessel diameters ($P < 0.05$; **Figures 5A–C**, **Supplementary Table 3**, **Supplementary Figure 3**). Of these, *V. berlandieri* × Novo Mexicana and *V. rupestris* had significantly lower vessel density, along with *V. vinifera* × *V. rupestris* ($P < 0.05$; **Figures 5A–C**, **Supplementary Table 3**, **Supplementary Figure 3**). *Vitis riparia, V. riparia* × *V. rupestris, V. berlandieri* × *V. riparia, V. berlandieri* × *V. rupestris, V. vinifera* × (*V. riparia* × *V. rupestris)* and *V. vinifera* × *V. berlandieri* all had a statistically different vessel surface ($P < 0.05$; **Figure 5C**, **Supplementary Table 3**).

Relationship Between Pathogen Tolerance and Xylem Anatomy

The effect of each xylem parameter classes was strong on *Pa. chlamydospora* DNA concentration ($P < 0.01$; **Figures 6A–C**,

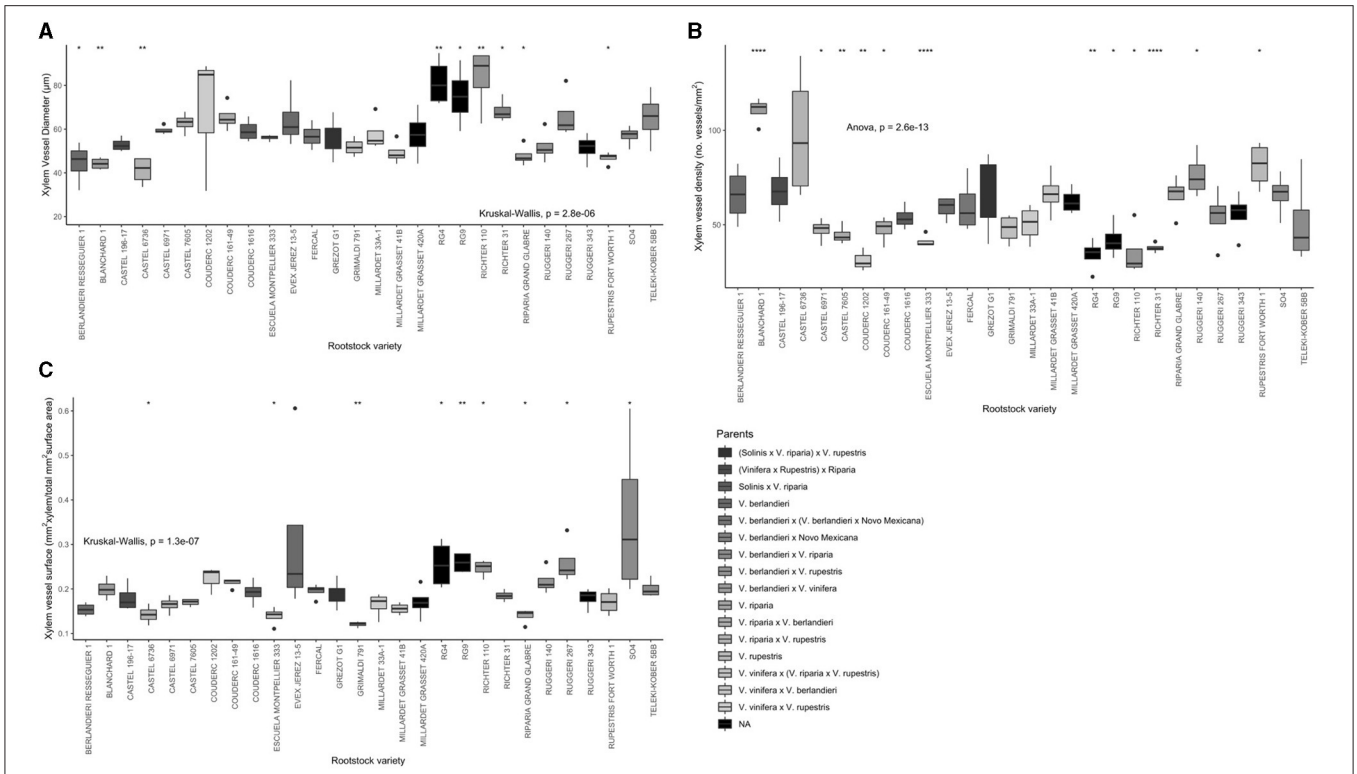

FIGURE 3 | Box plots of the histological traits for each rootstock variety. **(A)** Xylem vessel diameter (µm). **(B)** Xylem vessel density (number of vessels/mm^2 xylem). **(C)** Proportion of stem surface area covered by xylem vessels (mm^2 xylem vessels/total mm^2 vascular surface area). Significance (*$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$) determined by the Wilcoxon rank sum test for **(A,C)** and unpaired *T*-test for **(B)** ($P < 0.05$). Gray-scale color coded by rootstock parent cross (Solinis = *V. longii*).

Supplementary Figure 2). A marked effect of xylem vessel diameter and xylem density classes was also observed on *Pm. minimum* DNA concentration, but xylem vessel surface classes had no marked effect ($P < 0.01$; **Figures 6A–C**). A significantly higher *Pa. chlamydospora* DNA concentration was related to the rootstocks with xylem vessel diameters from 45 to 74 µm. The *Pa. chlamydospora* DNA concentration was also significantly higher for the rootstocks with xylem density values above 37 vessels/mm^2, and xylem surface values falling within the range of 0.2–0.24 mm^2 xylem/mm^2 total surface area (**Figures 6A–C**, **Supplementary Tables 4–6**). For *Pm. minimum*, the DNA concentration was significantly higher for the rootstocks with a vessel diameter above 75 µm and the xylem densities above 60 vessels/mm^2 (**Figures 6A–C**, **Supplementary Tables 7, 8**).

DISCUSSION

This study evaluated the relationship between the xylem anatomy of grapevine rootstocks and their susceptibility to *Pa. chlamydospora* and *Pm. minimum*. This is the first time that this interaction has been studied for *Pm. minimum*. Our results demonstrate the existence of a relationship between xylem anatomy and the ability of both *Pa. chlamydospora* and *Pm. minimum* to differentially colonize the vascular system of a wide range of grapevine rootstocks. We found significant differences between rootstocks for the DNA concentration of both pathogens detected in the inoculated shoots and for the estimated xylem parameters. A significant relation was observed between xylem vessel diameter and density, and the *Pa. chlamydospora* and *Pm. minimum* DNA concentrations. These results also agree with previous research that has studied the xylem vessel diameter effect on compartmentalization efficiency and thus, vascular pathogen movement, using the *V. vinifera-Pa. chlamydospora* interaction as a model system (Pouzoulet et al., 2017).

In our study, the susceptibility characterization of grapevine rootstocks was performed by assessing pathogen colonization based on traditional isolation methods in culture medium and DNA quantification by qPCR. The results showed significant differences for the disease incidence between rootstocks by following quantitative methods to estimate the amount of pathogen DNA while no significant differences were observed based on pathogen isolation. Colonization assessment can be hindered by techniques that rely on pathogen isolation from culturing tissue sections on nutritive media. In this context, only the accurate quantification of pathogen biomass can be useful to gain a better understanding of the disease reaction of host genotypes (Gramaje et al., 2013). In a previous study about grapevine tolerance to *Pa. chlamydospora*, fungal colonization was evaluated based on both wood necrotic lesion length and qPCR on inoculated plants (Pouzoulet et al., 2017). Similar

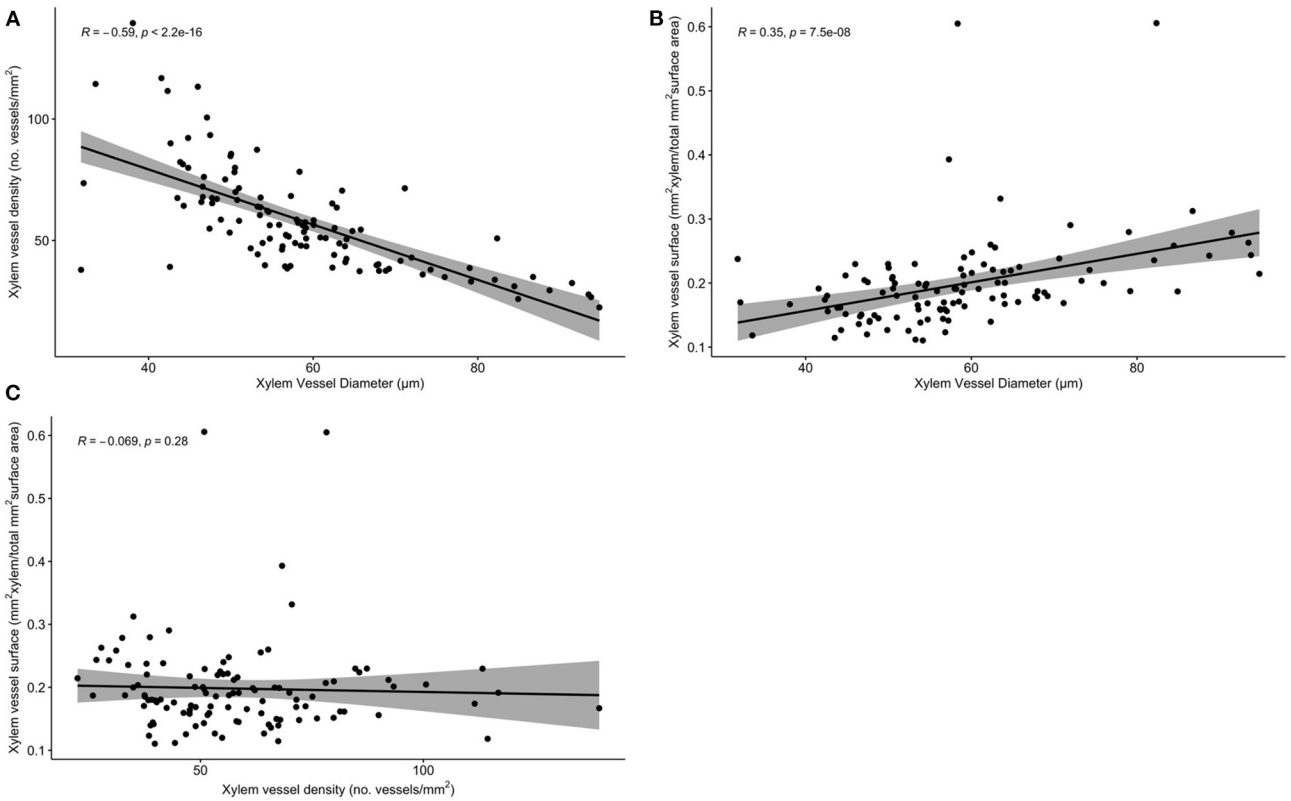

FIGURE 4 | Correlation plots between histological traits using the Kendall rank correlation coefficient. **(A)** Xylem vessel diameter correlated with xylem vessel density. **(B)** Xylem vessel diameter correlated with xylem vessel surface area. **(C)** Xylem vessel density correlated with xylem vessel surface area.

analyses have been performed for other perennial plants, such as olive, where the amount of pathogen DNA quantified in different genotypes correlated with susceptibility to vascular pathogen *Verticillium dahliae* (Mercado-Blanco et al., 2003).

Our results showed a significant effect of the xylem vessel diameter of the analyzed rootstocks on *Pa. chlamydospora* and *Pm. minimum* DNA concentrations. Significantly higher *Pa. chlamydospora* concentrations were detected for the diameter range from 55 to 64 µm and surface within the range of 0.2–0.24 mm^2 xylem vessels/mm^2. The *Pm. minimum* DNA concentration was significantly higher for a xylem density of above 66 vessels/mm^2. Some rootstocks like Berlandieri Resseguier 1 and Castel 6736, in which significantly lower pathogen DNA concentrations were detected, also showed significantly narrower vessel diameters.

Previous studies about the grapevine xylem anatomy on cultivars Merlot, Chardonnay, Cabernet Sauvignon, and Thompson Seedless have reported varying xylem vessel diameters and a positive correlation between the number of vessels with diameters above 120 µm and the *Pa. chlamydospora* DNA concentration (Pouzoulet et al., 2017). In general, less efficient pathogen movement restriction was observed for the cultivars proportionally harboring more vessels with a wide diameter, such as Thompson seedless, than for those like Merlot that proportionally displayed narrower diameter vessels (Pouzoulet et al., 2017). The herein obtained conclusions agree with previous reports that consider Merlot to be relatively resistant and Thompson seedless to be more susceptible to esca (Feliciano et al., 2004; Bruez et al., 2013; Murolo and Romanazzi, 2014).

Similar results were obtained when the same cultivars were assessed for Pierce's disease susceptibility (Deyett et al., 2019). From the herein obtained results, it can be speculated that cultivars with wide xylem vessels can be more prone to Pierce's disease decline caused by vascular pathogen *Xylella fastidiosa* (Deyett et al., 2019). Merlot has also been described as being tolerant to other diseases like Flavescence dorée given its ability to compartmentalize vascular vessels by forming tyloses, and also for its higher proportion of narrow vessels (Jelmini et al., 2020).

The relationship between xylem characteristics and the ability of either *Pa. chlamydospora* or *Pm. minimum* to colonize grapevine rootstocks would be useful to better understand the mechanisms associated with GTDs resistance. As indicated in previous studies, this relationship contributes to the hypothesis that a larger xylem vessel would be harder to obstruct (Pouzoulet et al., 2017, 2020a). One key determinant of plant resistance to vascular infections lies in the ability of the host to successfully compartmentalize invaders at the xylem level. Growing evidence supports the notion that the structural properties of the vascular system impact the vulnerability of the host to vascular pathogens

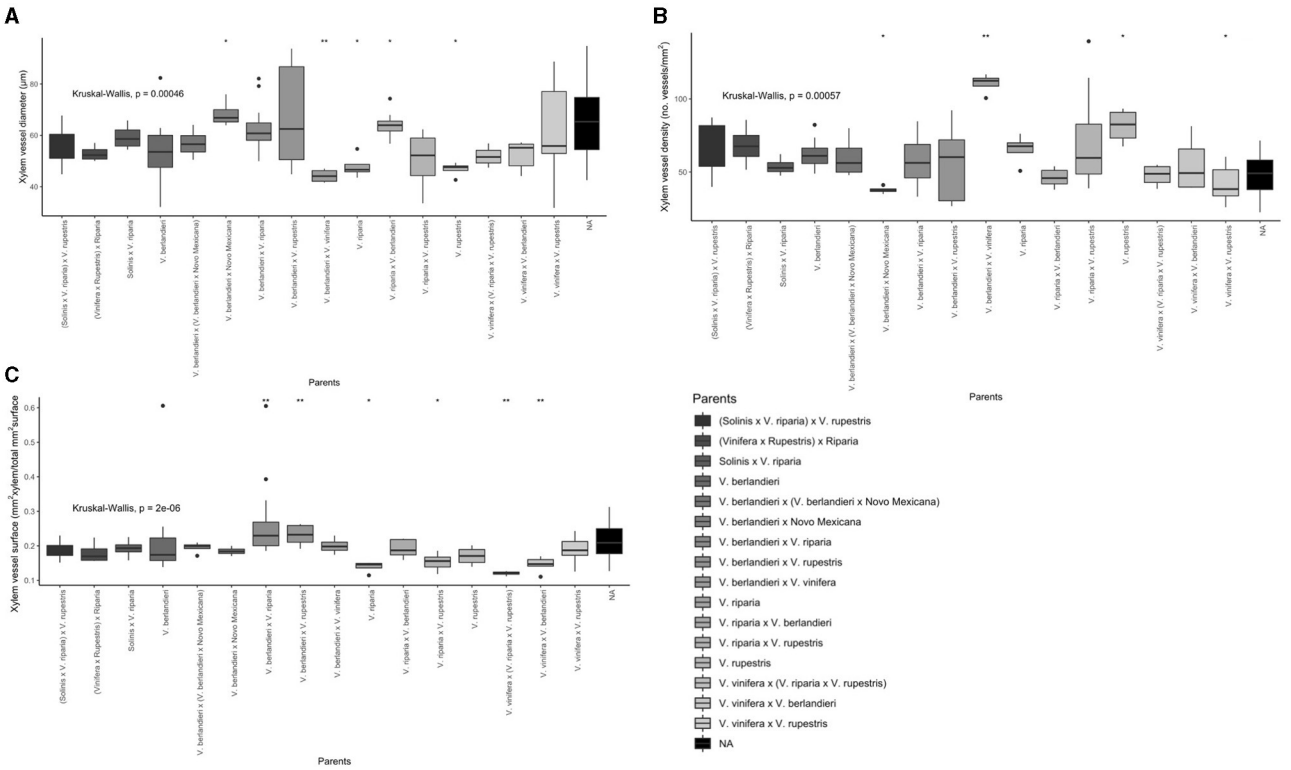

FIGURE 5 | Box plots of the histological traits for each rootstock parent cross (Solinis = *V. longii*). **(A)** Xylem vessel diameter (μm). **(B)** Xylem vessel density (number of vessels/mm^2 xylem). **(C)** Proportion of stem surface area covered by xylem vessels (mm^2 xylem vessels/total mm^2 vascular surface area). Significance (*$P < 0.05$, **$P < 0.01$) determined by the Wilcoxon rank sum test.

(Solla and Gil, 2002; Venturas et al., 2014; Pouzoulet et al., 2017, 2020a).

Recent studies further demonstrate how the grapevine xylem vessel diameter affects *Pa. chlamydospora* susceptibility (Pouzoulet et al., 2020a). In this context, the experimental xylem vessel diameter data of a single Cabernet Sauvignon genotype inoculated with *Pa. chlamydospora* has been used to calibrate a mechanistic stochastic model of pathogen spread and has evidenced that the efficiency of the compartmentalization process in a given xylem vessel is in accordance with its diameter (Pouzoulet et al., 2020a).

The importance of xylem anatomy to determine disease susceptibility has been previously described for other grapevine pathogens that systemically move through the vascular system, such as *X. fastidiosa* (Chatelet et al., 2011). Sun et al. (2013) hypothesized that vessels with wider diameters that harbor many tyloses might be linked with Pierce's disease symptom severity. The role of xylem anatomy in resistance to other pathogens that colonize the vascular system in perennial plants has also been reported for Dutch elm disease. Previous results have shown that a high proportion of vessels above 100 mm in diameter negatively correlate with host disease resistance (Solla and Gil, 2002; Venturas et al., 2014).

Disease tolerance of grapevine rootstocks to *Pa. chlamydospora* and *Pm. minimum* has been assessed by artificial inoculation based on *in vitro*, greenhouse, and field experiments with variable and contradictory results. For example, rootstock Richter 110 was rated as being susceptible under field and *in vitro* experimental conditions, but as tolerant when evaluated in greenhouse trials (Eskalen et al., 2001; Zanzotto et al., 2008; Gramaje et al., 2010). Rootstock 161-49C has been shown to be susceptible under greenhouse conditions and tolerant in field experiments (Eskalen et al., 2001; Gramaje et al., 2010). These variable results about susceptibility to these pathogens in the genetic grapevine pool could relate to their *Vitis* spp. cultivar pedigree. For example, it has been hypothesized that a variable degree of Pierce's susceptibility in *V. vinifera* could be partially attributed to the anatomical features of the host, which are shaped by its pedigree background (Deyett et al., 2019). In our study, significantly narrower vessel diameters were observed for rootstock parents *V. berlandieri* × *V. vinifera*, *V. riparia*, *V. riparia* × *V. rupestris*, and *V. rupestris*.

Anatomical measurements have also been linked with the adaptation of the xylem to dehydration in different grapevine cultivars (Pouzoulet et al., 2020b). Many xylem vessels and a large lumen area have been consistently associated with higher hydraulic conductivity and greater vulnerability to drought-induced cavitation (Pouzoulet et al., 2020b). Moreover, such cultivar conditions are consistent with domestication in a semi-arid habitat where a larger number and bigger size

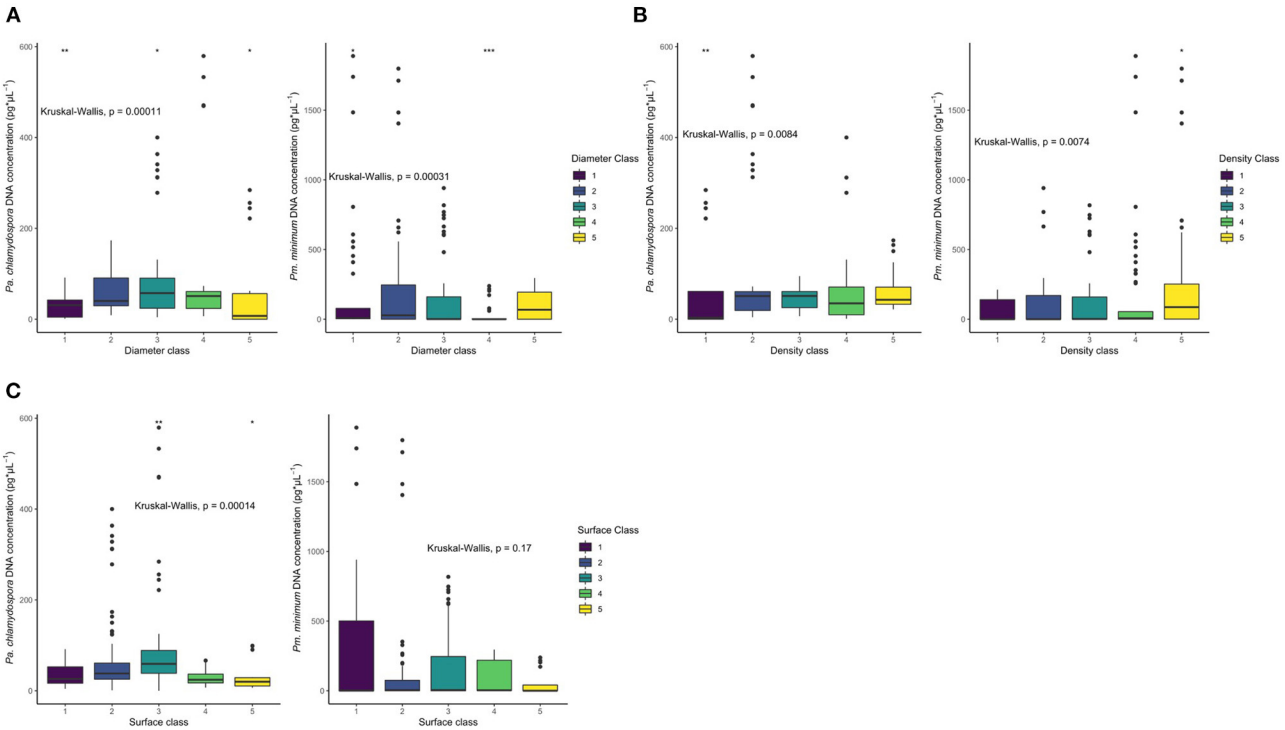

FIGURE 6 | Box plots of the fungal DNA concentration for each histological group. **(A)** The fungal DNA concentration in pg/µl for each diameter class (<44, 45–54, 55–64, 65–74, and 75–84 µm, respectively). Left: *Pa. chlamydospora*. Right: *Pm. minimum*. Color-coded by diameter class. **(B)** The fungal DNA concentration in pg/µl for each density class [<36, 37–46, 47–56, 57–66, 67, and above (vessels/mm^2), respectively]. Left: *Pa. chlamydospore*. Right: *Pm. minimum*. Color-coded by density class. **(C)** The fungal DNA concentration in pg/µl for each surface class [0.10–0.14, 0.15–0.19, 0.20–24, 0.24–0.30, 0.31 and above (xylem/total mm^2), respectively]. Left: *Pa. chlamydospora*. Right: *Pm. minimum*. Color-coded by Surface class. Surface classes are non-significant for *Pm. minimum* ($P < 0.05$). Significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) determined by the Wilcoxon rank sum test.

diversity of xylem vessels would be needed to transport water and to meet evaporative demand as opposed to cultivars domesticated in temperate forest regions. Evolution under different environmental conditions and domestication practices would explain such different strategies for water transportation and xylem anatomy traits (Pouzoulet et al., 2020b). It is important to consider the relation between drought and pathogen resistance because pathogens have been implicated as a mortality agent in plants weakened from drought (McDowell et al., 2008; Sala, 2010).

Without standard inoculation methodologies for GTD susceptibility assessments, it is useful to identify characteristics to select materials with putative resistance. In this context, the use of xylem characteristics, such as vessel diameter and vessel density, could provide a screening method to identify candidate grapevine rootstocks for further resistance testing. However, further research will be necessary to identify additional environmental effects like the effect of water availability because it may influence xylem development. Morphological traits of the vascular system, such as vessel diameter, are known to present developmental plasticity that responds to environmental factors during plant growth (Lovisolo and Schubert, 1998; Munitz et al., 2018; Pouzoulet et al., 2020b).

In the context of an integrated disease management program against GTDs, growers planting tolerant grapevine cultivars would significantly increase disease control. By providing pathogen-specific data on rootstock susceptibility, growers will have the choice to select adequate planting material in areas where some pathogens are preponderant. The information herein provided will allow researchers to offer growers planting recommendations in the short term and to provide the building blocks for future long-term breeding programs.

DATA AVAILABILITY STATEMENT

The datasets and code generated for this study can be found in the following github repository https://github.com/cramsing/Ramsing-et-al.-Xylem-anatomy-.

AUTHOR CONTRIBUTIONS

DG, JAr, and MB contributed to conception and design of the study. DG and FC provided plant materials. SM and JAg provided the xylem anatomy data. CR organized the database

and performed the analysis. CR and MB wrote the manuscript. All authors contributed to read and revise the manuscript and approved the submitted version.

FUNDING

This research was founded by FEDER funding through a State Program of I + D + i oriented to the Challenges of Society (RTA2015-00015-C02-00), supported by The National Institute for Agricultural and Food Research and Technology (INIA). DG was supported by the Ramón y Cajal program, Spanish Government (RyC-2017-23098). This research has been developed as a result of a mobility stay funded by the Erasmus+ – KA1 Erasmus Mundus Joint Master Degrees Programme of the European Commission under the PLANT HEALTH Project.

ACKNOWLEDGMENTS

We thank Madeleine Sieberhagen, Julián Restrepo, Ester Abarquero, and Antonio Ramón for their technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.726461/full#supplementary-material

REFERENCES

Bertsch, C., Ramírez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., et al. (2013). Grapevine trunk diseases: complex and still poorly understood. *Plant Pathol.* 62, 243–265. doi: 10.1111/j.1365-3059.2012.02674.x

Bruez, E., Lecomte, P., Grosman, J., Doublet, B., Bertsch, C., Fontaine, F., et al. (2013). Overview of grapevine trunk diseases in France in the 2000s. *Phytopathol. Mediterr.* 52, 262–275. doi: 10.14601/Phytopathol_Mediterr-11578

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622. doi: 10.1373/clinchem.2008.112797

Chatelet, D. S., Wistrom, C. M., Purcell, A. H., Rost, T. L., and Matthews, M. A. (2011). Xylem structure of four grape varieties and 12 alternative hosts to the xylem-limited bacterium *Xylella fastidiosa*. *Ann. Botany* 108, 73–85. doi: 10.1093/aob/mcr106

Crous, P. W., and Gams, W. (2000). *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of *Petri grapevine* decline and esca. *Phytopathol. Mediterr.* 39, 112–188. Available online at: http://www.jstor.org/stable/26456536

de Mendiburu, F. (2019). *Agricolae: Statistical Procedures for Agricultural Research.* R package version 1.3-1. Available online at: https://CRAN.R-project.org/package=agricolae

Deyett, E., Pouzoulet, J., Yang, J.-I., Ashworth, V. E., Castro, C., Roper, M. C., et al. (2019). Assessment of Pierce's disease susceptibility in *Vitis vinifera* cultivars with different pedigrees. *Plant Pathol.* 68, 1079–1087. doi: 10.1111/ppa.13027

Eskalen, A., Gubler, W. D., and Khan, A. (2001). Rootstock susceptibility to *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. *Phytopathol. Mediterr.* 40, S433–S438. Available online at: http://www.jstor.org/stable/44981653

Feliciano, A. J., Eskalen, A., and Gubler, W. D. (2004). Differential susceptibility of three grapevine cultivars to *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* in California. *Phytopathol. Mediterr.* 43, 66–69. doi: 10.14601/Phytopathol_Mediterr-1727

Fradin, E. F., and Thomma, B. P. H. J. (2006). Physiology and molecular aspects of verticillium wilt diseases caused by *V. dahliae and V. albo-atrum. Mol. Plant Pathol.* 7, 71–86. doi: 10.1111/j.1364-3703.2006.00323.x

Gramaje, D., and Armengol, J. (2011). Fungal trunk pathogens in the grapevine propagation process: potential inoculum sources, detection, identification, and management strategies. *Plant Dis.* 95, 1040–1055. doi: 10.1094/PDIS-01-11-0025

Gramaje, D., García-Jiménez, J., and Armengol, J. (2010). Field evaluation of grapevine rootstocks inoculated with fungi associated with petri disease and esca. *Am. J. Enol. Viticult.* 61, 512–520. doi: 10.5344/ajev.2010.10021

Gramaje, D., Mostert, L., Groenewald, J. Z., and Crous, P. W. (2015). *Phaeoacremonium*: from esca disease to phaeohyphomycosis. *Fungal Biol.* 119, 759–783. doi: 10.1016/j.funbio.2015.06.004

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2013). A comparison of real-time PCR protocols for the quantitative monitoring of asymptomatic olive infections by Verticillium dahliae pathotypes. *Phytopathology* 103, 1058–1068. doi: 10.1094/PHYTO-11-12-0312-R

Gramaje, D., Urbez-Torres, J. R., and Sosnowski, M. R. (2018). Managing grapevine trunk diseases with respect to etiology and epidemiology: current strategies and future prospects. *Plant Dis.* 102, 12–39. doi: 10.1094/PDIS-04-17-0512-FE

Hofstetter, V., Buyck, B., Croll, D., Viret, O., Couloux, A., and Gindro, K. (2012). What if esca disease of grapevine were not a fungal disease? *Fungal Div.* 54, 51–67. doi: 10.1007/s13225-012-0171-z

Jelmini, J., Rizzoli, A., Jermini, M., Schumpp, O., and Conedera, M. (2020). Phloem and xylem modifications of *Vitis vinifera* stems in response to flavescence dorée phytoplasma infection. *Plant Pathol.* 70, 970–979 doi: 10.1111/ppa.13330

Kassambara, A. (2020). *ggpubr: 'ggplot2' Based Publication Ready Plots.* R package version 0.4.0. Available online at: https://CRAN.R-project.org/package=ggpubr

Kay, M., and Wobbrock, J. (2020). *ARTool: Aligned Rank Transform for Nonparametric Factorial ANOVAs.* R package version 0.10.18.

Landi, L., Murolo, S., and Romanazzi, G. (2012). Colonization of *Vitis* spp. Wood by sGFP-transformed *Phaeomoniella chlamydospora*, a tracheomycotic fungus involved in esca disease. *Phytopathology* 102, 290–297. doi: 10.1094/PHYTO-06-11-0165

Lorena, T., Calamassi, R., Mori, B., Mugnai, L., and Surico, G. (2001). Phaeomoniella chlamydospora-grapevine interaction: Histochemical reactions to fungal infection. *Phytopathol. Mediterr.* 40, 400–406. doi: 10.14601/Phytopathol_Mediterr-1610

Lovisolo, C., and Schubert, A. (1998). Effects of water stress on vessel size and xylem hydraulic conductivity in *Vitis vinifera* L. *J. Experimental Bot.* 49, 693–700. doi: 10.1093/jxb/49.321.693

Martínez-Diz, M., del, P., Díaz-Losada, E., Barajas, E., Ruano-Rosa, D., Andrés-Sodupe, M., et al. (2019). Screening of Spanish *Vitis vinifera* germplasm for resistance to *Phaeomoniella chlamydospora*. *Sci. Horticult.* 246, 104–109. doi: 10.1016/j.scienta.2018.10.049

McDowell, N., Pockman, W. T., Allen, C. D., Breshears, D. D., Cobb, N., Kolb, T., et al. (2008). Mechanisms of plant survival and mortality during drought:why do some plants survive while others succumb to drought? *New Phytol.* 178, 719–739. doi: 10.1111/j.1469-8137.2008.02436.x

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiol. Mol. Plant Pathol.* 63, 91–105. doi: 10.1016/j.pmpp.2003.10.001

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., et al. (2018). Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Dis.* 102, 1189–1217. doi: 10.1094/PDIS-08-17-1181-FE

Mostert, L., Groenewald, J. Z., Summerbell, R. C., Gams, W., and Crous, P. W. (2006a). Taxonomy and pathology of *Togninia* (diaporthales) and its *Phaeoacremonium* anamorphs. *Stud. Mycol.* 54, 1–115. doi: 10.3114/sim.54.1.1

Mostert, L., Halleen, F., Fourie, P., and Crous, P. W. (2006b). A review of Phaeoacremonium species involved in Petri disease and esca of grapevines. *Phytopathol. Mediterr.* 45, 12–29. doi: 10.14601/Phytopathol_Mediterr-1844

Munitz, S., Netzer, Y., Shtein, I., and Schwartz, A. (2018). Water availability dynamics have long-term effects on mature stem structure in *Vitis vinifera*. *Am. J. Bot.* 105, 1443–1452 doi: 10.1002/ajb2.1148

Murolo, S., and Romanazzi, G. (2014). Effects of grapevine cultivar, rootstock and clone on esca disease. *Australas. Plant Pathol.* 43, 215–221. doi: 10.1007/s13313-014-0276-9

Nelson, R., Wiesner-hanks, T., Wisser, R., and Balint-kurti, P. (2018). Navigating complexity to breed disease-resistant crops. *Nat. Rev. Genet.* 19, 21–33. doi: 10.1038/nrg.2017.82

Niks, R. E., Qi, X. Q., and Marcel, T. C. (2015). Quantitative resistance to biotrophic filamentous plant pathogens: concepts, misconceptions and mechanisms. *Annu. Rev. Phytopathol.* 69, 181–196. doi: 10.1146/annurev-phyto-080614-115928

Ogle, D. H., Wheeler, P., and Dinno, A. (2021). *FSA: Fisheries Stock Analysis.* R package version 0.8.32. Available online at: https://github.com/droglenc/FSA.

Pierron, R., Gorfer, M., Berger, H., Jacques, A., Sessitsch, A., Strauss, J., et al. (2015). Deciphering the niches of colonisation of *Vitis vinifera* L. by the esca-associated fungus *Phaeoacremonium aleophilum* using a gfp marked strain and cutting systems. *PLoS ONE* 10:e0126851. doi: 10.1371/journal.pone.0126851

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C., and Nelson, R. J. (2008). Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* 14, 21–29 doi: 10.1016/j.tplants.2008.10.006

Pouzoulet, J., Mailhac, N., Couderc, C., Besson, X., Dayd,é, J., Lummerzheim, M., et al. (2013). A method to detect and quantify *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* DNA in grapevine-wood samples. *App. Microbiol. Biotech.* 97, 10163–10175. doi: 10.1007/s00253-013-5299-6

Pouzoulet, J., Pivovaroff, A. L., Santiago, L. S., and Rolshausen, P. E. (2014). Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from dutch elm disease and esca disease in grapevine. *Front. Plant Sci.* 5:253. doi: 10.3389/fpls.2014.00253

Pouzoulet, J., Pivovaroff, A. L., Scudiero, E., De Guzmann, M. E., Rolshausen, P. E., and Santiago, L. S. (2020b). Contrasting adaptation of xylem to dehydration in two *Vitis vinifera* L. sub-species. *Vitis* 59, 53–61. doi: 10.5073/vitis.2020.59.53-61

Pouzoulet, J., Rolshausen, P., Charbois, R., Chen, J., Guillaumie, S., Ollat, N., et al. (2020a). Behind the curtain of the compartmentalization process: exploring how xylem vessel diameter impacts vascular pathogen resistance. *Plant Cell Environ.* 43, 2782–2796. doi: 10.1111/pce.13848

Pouzoulet, J., Scudiero, E., Schiavon, M., and Rolshausen, P. E. (2017). Xylem vessel diameter affects the compartmentalization of the vascular pathogen *Phaeomoniella chlamydospora* in grapevine. *Front. Plant Sci.* 5:1442. doi: 10.3389/fpls.2017.01442

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: https://www.R-project.org/

Saito, S., Dunne, K. J., Evans, K. J., Barry, K., Cadle-Davidson, L., and Wilcox, W. F. (2013). Optimisation of techniques for quantification of *Botrytis cinerea* in grape berries and receptacles by quantitative polymerase chain reaction. *Austr. J. Grape Wine Res.* 19, 68–73. doi: 10.1111/ajgw.12011

Sala, A. (2010). Physiological mechanisms of drought-induced tree mortality are far from being resolved. *New Phytol.* 186, 274–281 doi: 10.1111/j.1469-8137.2009.03167.x

Solla, A., and Gil, L. (2002). Xylem vessel diameter as a factor in resistance of *Ulmus minor* to *Ophiostoma novo-ulmi*. *Forest Pathol.* 32, 123–134. doi: 10.1046/j.1439-0329.2002.00274.x

Sosnowski, M., Ayres, M., McCarthy, M., Wicks, T., and Scott, E. (2016). Investigating the potential for resistance to grapevine trunk diseases. *Wine Vitic. J.* 31, 41–45. Available online at: https://winetitles.com.au/wvj/articles/wine-viticulture-journal-volume-31-no-5-2016/investigating-the-potential-for-resistance-to-grapevine-trunk-diseases/

St. Clair, D. A. (2010). Quantitative disease resistance and quantitative resistance loci in breeding. *Ann. Rev. Phytopathol.* 48, 247–268. doi: 10.1146/annurev-phyto-080508-081904

Sun, Q., Sun, Y. L., Walker, M. A., and Labavitch, J. M. (2013). Vascular occlusions in grapevines with Pierce's disease make disease symptom development worse. *Plant Phys.* 161, 1529–1541. doi: 10.1104/pp.112.208157

Suthman, D. D., Leonard, K. J., and Miller-Garvin, J. (2007). "Breeding crops for durable resistance to disease," *Adv. Agron.* 95, 319–367. doi: 10.1016/S0065-2113(07)95004-X

Venturas, M., López, R., Martín, J. A., Gascó, A., and Gil, L. (2014). Heritability of *Ulmus minor* resistance to Dutch elm disease and its relationship to vessel size, but not to xylem vulnerability to drought. *Plant Pathol.* 63, 500–509. doi: 10.1111/ppa.12115

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis.* Springer-Verlag R package version 3.2.1. Available online at: https://CRAN.R-project.org/package=ggplot2

Wickham, H., François, R., Henry, L., and Müller, K. (2020). *dplyr: A Grammar of Data Manipulation.* R package version 1.0.2. Available online at: https://CRAN.R-project.org/package=dplyr

Zanzotto, A., Gardiman, M., and Lovat, L. (2008). Effect of *Phaeomoniella chlamydospora* and *Phaeoacremonium* sp. on *in vitro* grapevine plants. *Sci. Horticult.* 116, 404–408. doi: 10.1016/j.scienta.2008.03.002

Conflict of Interest: 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: 20 October 2021
doi: 10.3389/fpls.2021.762889

Compatibility Evaluation and Anatomical Observation of Melon Grafted Onto Eight *Cucurbitaceae* Species

*Mu Xiong, Changjin Liu, Liping Guo, Jin Wang, Xiangshuai Wu, Ling Li, Zhilong Bie and Yuan Huang**

Key Laboratory of Horticultural Plant Biology, Ministry of Education, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, China

Melon (*Cucumis melo*) is one of the top 10 fruits in the world, and its production often suffers due to soil-borne diseases. Grafting is an effective way to solve this problem. However, graft incompatibility between scion and rootstock limits the application of melon grafting. In this study, the melon was grafted onto eight *Cucurbitaceae* species (cucumber, pumpkin, melon, luffa, wax gourd, bottle gourd, bitter gourd, and watermelon), and graft compatibility evaluation and anatomical observation were conducted. Taking melon homo-grafted plants as control, melon grafted onto cucumber and pumpkin rootstocks was compatible, while melon grafted onto luffa, wax gourd, bottle gourd, bitter gourd, and watermelon rootstocks was incompatible based on the scion dry weight on day 42 after grafting. Meanwhile, we found that starch–iodine staining of scion stem base is an index to predict graft compatibility earlier, on day 14 after grafting. Further, microsection observations showed that there was more cell proliferation at graft junction of melon hetero-grafted combinations; vascular reconnection occurred in all graft combinations. However, excess callose deposited at graft junction resulted in the blockage of photosynthate transport, thus, leading to starch accumulation in scion stem base, and finally graft incompatibility. In addition, undegraded necrotic layer fragments were observed at graft junctions of melon grafted onto incompatible bitter gourd and watermelon rootstocks. The above results provide clues for the selection and breeding of compatible *Cucurbitaceae* rootstocks of melon and demonstrate that starch accumulation in scion base and callose deposition at graft junction is associated with melon graft compatibility.

Keywords: melon, graft compatibility, *Cucurbitaceae*, starch accumulation, vascular development, callose deposition, necrotic layer

OPEN ACCESS

Edited by:
Vittorino Novello, University of Turin, Italy

Reviewed by:
Muhammad Azher Nawaz, University of Sargodha, Pakistan
Phatu William Mashela, University of Limpopo, South Africa
Athanasios Koukounaras, Aristotle University of Thessaloniki, Greece

****Correspondence:***
Yuan Huang
huangyuan@mail.hzau.edu.cn

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

Received: *23 August 2021*
Accepted: *20 September 2021*
Published: *20 October 2021*

Citation:
Xiong M, Liu C, Guo L, Wang J, Wu X, Li L, Bie Z and Huang Y (2021) Compatibility Evaluation and Anatomical Observation of Melon Grafted Onto Eight Cucurbitaceae Species. Front. Plant Sci. 12:762889. doi: 10.3389/fpls.2021.762889

INTRODUCTION

Grafting, as an asexual plant propagation technology, has been applied for about 3000 years (Melnyk and Meyerowitz, 2015). It is widely used in agriculture as it enhances abiotic/biotic stress resistance, extends the harvesting period, improves fruit yield and quality, adjusts flowering time, and improves fruit tree architecture (Lee et al., 2010; Louws et al., 2010; Huang et al., 2011;

Nawaz et al., 2016, 2017; Ceballos et al., 2017; Niu et al., 2018; Souza et al., 2018; Zhong et al., 2018; Gautier et al., 2019; Migicovsky et al., 2019). However, graft incompatibility usually occurs between scion and rootstock, leading to a low survival ratio, abnormal growth, and low yield (Ren et al., 2018).

Graft incompatibility has been reported in various horticultural species. When watermelon was grafted onto different pumpkin rootstocks, the survival rate of graft incompatible combinations was significantly reduced (Yetisir and Sari, 2003; Yetisir et al., 2007). Litchi scion leaves turned yellow and the scion base swelled 6 weeks after being grafted onto incompatible rootstocks (Chen Z. et al., 2016, 2017). The survival ratio could reach 87 and 90% on day 30 but reduced to 0 and 7% on day 180 after honey pomelo (*Citrus grandis*) cv. 'Hongmianmiyou' and 'Huangjinmiyou' were grafted onto *Poncirus trifoliata* (Gong et al., 2016). Tomato/pepper healing junction showed the asynchronous stem bulging 30 days after grafting (Masayuki et al., 2000; Thomas et al., 2021), which was also observed in graft incompatible melon (Cohen et al., 2007; Zhou et al., 2018).

Melon (*Cucumis melo* L.) is an important horticultural crop that belongs to the *Cucurbitaceae* family. Melon often suffers due to soil-borne pathogens. Intraspecific grafting in a melon can be used to avoid damage caused by wilt pathogens, without loss of yield and fruit quality, but cannot suppress root and stem rot diseases (Cohen et al., 2007). Pumpkin rootstocks provide non-specific and efficient protection against those pathogens, and against some abiotic stresses (Cohen et al., 2007; Shang et al., 2016). The differences between melon scion and pumpkin rootstock in water absorption and sugar distribution were not correlated with graft incompatibility on day 14 but on day 24 after grafting (Aloni et al., 2008). The problem of an unbalanced distribution of sugar was also reported by Camalle et al. (2021), who found that there was over-accumulation of sugars and sugar alcohols in the scion of melon grafted onto incompatible pumpkin rootstock (Ki/r53), as compared with compatible pumpkin homo-grafted combination (r53/r53). However, the *Cucurbitaceae* family contains ∼1000 species, which includes many important vegetables and fruits, such as cucumber, melon, wax gourd, watermelon, pumpkin, bitter gourd, luffa, and bottle gourd (Guo et al., 2020). Previous studies on melon graft compatibility were limited to several rootstock species, such as pumpkin and bottle gourd, and a comprehensive evaluation and anatomical observation of compatibility between melon and a range of *Cucurbitaceae* species are lacking.

Successful vascular reconnection is regarded as a landmark event of graft healing (Melnyk, 2017). In amaranth/tomato (Xiang et al., 1992), soybean/pumpkin (Sun et al., 2015), arabidopsis/tomato (Flaishman et al., 2008), and arabidopsis/chrysanthemum (Notaguchi et al., 2020), the persistent necrotic layer of graft junction prevented the vascular bundle differentiation and reconnection, resulting in graft failure. When cucumber was grafted onto incompatible pumpkin rootstocks, its necrotic layer disappeared later than when grafted onto compatible rootstocks (Xu et al., 2016). However, after arabidopsis was grafted onto cabbage, radish, and tobacco, callus was formed at the graft junction, but there were few complete vascular bundles (Flaishman et al., 2008; Notaguchi et al., 2020). The vascular bundles of incompatible grafted combinations in litchi reconnected normally at the early stage, but gaps were formed at the later stage (Chen Z. et al., 2016, 2017). Meanwhile, Thomas et al. (2021) observed parenchymatous callus formation at the junction of compatible homo-grafted tomato and pepper, and incompatible hetero-grafted tomato/pepper, and pepper/tomato; however, the delayed vascular progression and xylem discontinuity occurred in the incompatible hetero-grafted tomato/pepper, and pepper/tomato combinations. Graft incompatibility also affects the xylem and/or the phloem functionality, and hence, the bidirectional transport of photoassimilates, hormones, mineral nutrients, and water is negatively affected (Schoning and Kollmann, 1997; Espen et al., 2005; Kawaguchi et al., 2008; Camalle et al., 2021). The transport of photoassimilates to the roots was blocked on day 78 after grafting in incompatible peach/plum combinations (Espen et al., 2005). In *in vitro* hetero-grafts, the non-transporting sieve-tubes were observed at the junction of *Vicia* grafted onto *Helianthus* (Schoning and Kollmann, 1997). Thus, it is essential to observe the anatomical structure of the graft junction of melon grafted onto different species.

In this study, we evaluated the graft compatibility of melon grafted onto eight *Cucurbitaceae* species and found that starch–iodine staining was a key index, which could be used to predict graft compatibility earlier. Excess callose was deposited in newborn phloem of incompatible combinations, leading to starch accumulations in the scion base. Undegraded necrotic layer fragments were observed at the graft junction of melon grafted onto incompatible bitter gourd and watermelon rootstocks. This study provides useful clues for the selection and breeding of melon rootstock, and more insights to understand the graft compatibility mechanism of melon.

MATERIALS AND METHODS

Plant Material and Grafting

In this study, eight *Cucurbitaceae* species (cucumber, pumpkin, melon, luffa, wax gourd, bottle gourd, bitter gourd, and watermelon) including 40 cultivars were used to evaluate the melon graft compatibility. A Xinjiang local cultivar, 'Akekekouqi,' was used as the melon scion. The detailed information on these cultivars is provided in **Supplementary Table 1**.

The experiments were conducted in 2021 at the National Center of Vegetable Improvement in Huazhong Agricultural University, Central China (30°27′N, 114°20′E, and altitude 22 m above sea level). The seeds were sown in the 50-cell plug trays. Seedlings were cultivated with a day/night (14/10 h) cycle at 28/18°C, and 60–70% relative humidity, in a climate chamber. When the scion cotyledons had fully opened and the first true leaf of rootstocks had fully unfolded, one cotyledon grafting was performed as described by Hassell et al. (2008). The grafted

seedlings were then maintained under the conditions described previously (Liu et al., 2021). Briefly, the grafted seedlings were maintained under complete darkness on day 1, low light intensity (80 μmol m^{-2} s^{-1}, 14/10 h photoperiod) from day 2 to day 7, and normal light intensity (170 μmol m^{-2} s^{-1}, 14/10 h photoperiod) from day 7. The light source was a full-spectrum LED tube light T8 (Gexinlai Optoelectronics Technology Co., Ltd., China). The temperatures were kept at 28/18°C (day/night) during graft healing. The humidity was kept above 95% during the first 5 days, and decreased to 85% from day 6 to day 10, and then to 70% from day 10. For long-term evaluation experiments, grafted seedlings were transplanted into plastic pots on day 14 after grafting, each containing 10 L of the substrate (peat: vermiculite: perlite = 1:1:1, v/v). Each pot contained one grafted seedling. The pots were arranged at 150 cm row spacing, spaced 50 cm apart in a greenhouse, from April to May 2021. The average day/night temperature was 28/18°C. Plants were irrigated with a full-strength Hoagland nutrient solution.

Determination of Scion Dry Weight, Scion Height, and Scion and Rootstock Stem Diameters

For scion dry weight determination, three plants per replicate were harvested on day 42 after grafting (28 days after transplanting). The scion samples were dried in a forced-air oven at 85°C for 96 h and then weighted. Scion height and scion and rootstock diameters were determined on day 14, day 21, day 28, and day 42 after grafting. Scion and rootstock diameters were determined at 5 mm above or below the graft junction. Stem diameter ratio = (scion diameter/rootstock diameter).

Determination of Leaf Area and Root Length Ratio, and Leaf SPAD

The 14-day-old grafted plants were analyzed. Leaf area ratio = (true leaf area of melon hetero-grafted plants/true leaf area of melon homo-grafted plants). Root length ratio = (root length of melon hetero-grafted plants/root length of rootstock homo-grafted plants). Leaf area and root length were determined by WinRHIZO (Regent, Canada). SPAD (relative chlorophyll value) was determined using the second true leaf from the top with SPAD-502 Chlorophyll Meter (Konica Minolta, Japan).

Starch–Iodine Staining of Scion and Rootstock Stem

Plants were sampled on day 14 and day 42 after grafting. To conduct the starch–iodine staining, 2 mm stem above and below the graft junction were cut as the scion and rootstock respectively. The collected samples were placed in 75% ethanol for 24 h to decolorize. After washing the decolorized samples using ddH_2O_2 for 1 min, they were placed in I_2–KI solution (24 mg I_2, 96 mg KI/ml) for 1 min, followed by washing with ddH_2O_2 for 1 min. The stained stem was imaged using a stereoscopic microscope (Olympus SZ61, Olympus, Japan). Starch–iodine staining area ratio = (staining area/transected stem area). The area was analyzed using ImageJ[1].

Anatomical Observation of Graft Junction

Graft junctions were collected on day 14 after grafting. The collected samples were placed in 70% FAA (Formaldehyde-acetic acid-ethanol Fixative, Formaldehyde: Acetic acid: Ethanol = 1: 1: 18, volume ratio) for 24 h, and then stored in 70% ethanol at 4°C. The paraffin section was performed as described by El-Gazzar et al. (2017). Samples were sectioned to 8 μm vertically using a rotary microtome (Leica RM2255, Leica, Germany), dewaxed, rehydrated, cleaned, stained with 1% safranin, counterstained with 1% fast green, and then fixed with neutral balata. Sections were imaged using a positive fluorescence microscope (Leica DM6B, Leica, Germany). The vibratome section was performed as described by Wang et al. (2018). Samples were embedded as tissues into 4% agarose and sectioned to 100 μm vertically using an automatic vibratome (Leica VT1200S, Leica, Germany). Lignin was stained for 5 min using 0.01% Basic fuchsin and imaged using a confocal laser scanning microscope (Leica SP8, Leica, Germany). Callose was stained for 1 h using 0.01% aniline blue in 150 mM KH_2PO_4 (pH = 9.5) without light and imaged using a confocal laser scanning microscope (Leica SP8, Leica, Germany). For lignin, fluorescence was detected at 552 nm excitation, 580–630 nm emission wavelength; for callose, fluorescence was detected at 405 nm excitation, 505–545 nm emission wavelength.

Statistical Analysis

All data were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL, United States). The significance analysis was performed using Student's *t*-test ($p < 0.05$). The correlation analysis was done using the Pearson method. The column diagram was made using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, Canada). The heat map of correlation analysis was made by R.[2]

RESULTS

Scion Dry Weight of Melon Grafted Onto Eight *Cucurbitaceae* Species

Compared with melon homo-grafted plants, hetero-grafted rootstocks affected the scion dry weight (**Figure 1**). According to scion dry weight, cucumber and pumpkin were graft compatible rootstocks, and for cucumber rootstocks, four out of six cultivars had significantly higher scion dry weight than melon homo-grafted plants and, therefore, had higher compatibility than pumpkin rootstocks (**Figure 1B**). By contrast, luffa, wax gourd, bottle gourd, bitter gourd, and watermelon rootstocks were graft incompatible with melon, and scion dry weight of all tested cultivars was significantly lower than melon homo-grafted plants (**Figure 1B**).

[1]https://imagej.nih.gov/ij/

[2]https://www.r-project.org/

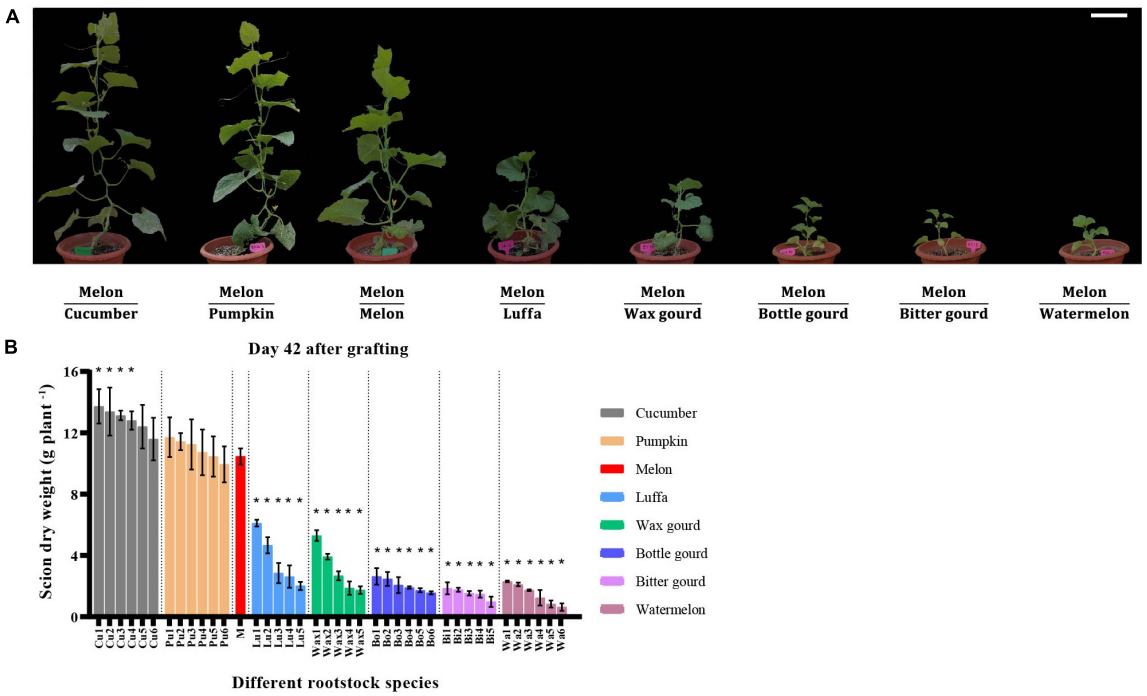

FIGURE 1 | Pictures and scion dry weight of melon grafted onto eight *Cucurbitaceae* species. **(A)** Pictures of melon cv. 'Akekekouqi' grafted onto cucumber cv. 'Jinyou No.35,' pumpkin cv. 'Qingyouzhen No.1,' melon cv. 'Akekekouqi,' luffa cv. 'Sanbi No.6,' wax gourd cv. 'Aonong,' bottle gourd cv. 'H19,' bitter gourd cv. 'Liangku No.1,' and watermelon cv. 'Zaojia 8424' on day 42 after grafting. Scale bar represents 15 cm. **(B)** Scion dry weight of melon cv. 'Akekekouqi' grafted onto cucumber (Cu1–Cu6), pumpkin (Pu1–Pu6), melon (M), luffa (Lu1–Lu5), wax gourd (Wax1–Wax5), bottle gourd (Bo1–Bo6), bitter gourd (Bi1–Bi5), and watermelon (Wa1–Wa6) on day 42 after grafting. Asterisks indicate a significant difference between melon homo-grafted plants and hetero-grafted plants on day 42 after grafting using Student's *t*-test ($p < 0.05$).

Scion Height and Scion and Rootstock Stem Diameters of Melon Grafted Onto Eight *Cucurbitaceae* Species

Melon grafted onto cucumber rootstocks had significantly higher scion height than melon homo-grafted plants on day 14, day 21, day 28, and day 42 after grafting, and only Cu5 rootstock showed no significant difference on day 14 (**Figure 2**). There was a significant difference in scion height of melon grafted onto different pumpkin rootstocks, but no hetero-grafted combination showed a significantly lower height as compared with melon homo-grafted plants (**Figure 2**). Luffa, wax gourd, bottle gourd, and watermelon rootstocks showed significance on day 14, day 21, and day 28, but no hetero-grafted combination showed a significantly higher scion height as compared with melon homo-grafted plants (**Figures 2A–C**); and the scion height of all cultivars of these rootstocks was significantly lower on day 42 (**Figure 2D**). Bitter gourd 1 and Bitter gourd 2 rootstocks showed significantly higher scion height than melon homo-grafted plants on day 14 and day 21; however, all cultivars showed significantly lower height on day 42 (**Figure 2**).

The stem diameter of graft junction was usually related to graft compatibility. On day 14 after grafting, the stem diameter ratio showed that five out of six cucumbers, four out of five luffas, four out of five wax gourds, three out of six bottle gourds, four out of five bitter gourd cultivars, and all watermelon rootstocks grafted combinations had thicker scion base than rootstock (**Figure 3A**). All rootstock species, excluding melon homo-grafted plants, showed a thicker scion base than rootstock on day 42; the watermelon rootstocks grafted combinations had the highest stem diameter ratio of scion/rootstock (**Figure 3D**). Furthermore, rootstock diameters of incompatible combinations were generally lower than compatible combinations on day 42 (**Figures 3A–D**; **Supplementary Figures 2, 3**).

Leaf Area and Root Length Ratio, and Leaf SPAD of Melon Grafted Onto Eight *Cucurbitaceae* Species

Compared with melon homo-grafted plants, hetero-grafted rootstocks had different effects on leaf area ratio, where only six cultivars of watermelon rootstocks showed a significantly lower ratio on day 14 after grafting (**Figure 4A**). Compared with rootstock homo-grafted plants, 15 cultivars of 39 hetero-grafted combinations, including one cucumber, two pumpkins, one luffa, three wax gourds, one bottle gourd, two bitter gourds, and five watermelons, had significant negative effects, and only four cultivars including two cucumbers, one luffa, and one bottle gourd had significantly positive effects on root length ratio on day 14 (**Figure 4B**). For SPAD, there was no significant

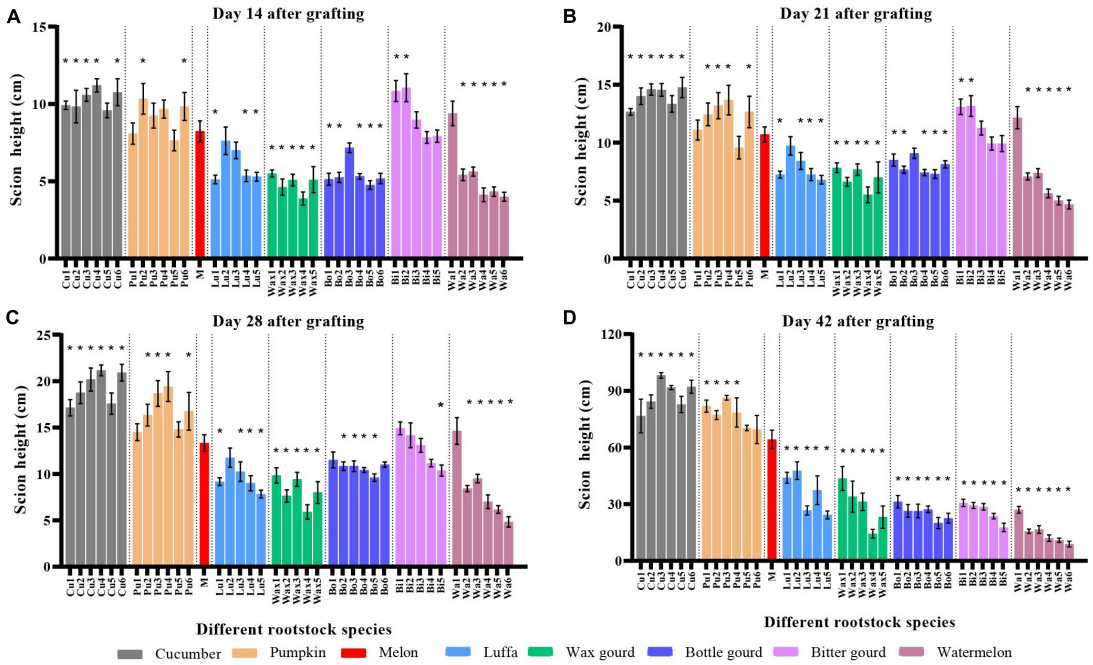

FIGURE 2 | Scion height of melon grafted onto eight *Cucurbitaceae* species. Scion height of melon cv. 'Akekekouqi' grafted onto cucumber (Cu1–Cu6), pumpkin (Pu1–Pu6), melon (M), luffa (Lu1–Lu5), wax gourd (Wax1–Wax5), bottle gourd (Bo1–Bo6), bitter gourd (Bi1–Bi5), and watermelon (Wa1–Wa6) on day 14 **(A)**, day 21 **(B)**, day 28 **(C)**, and day 42 **(D)** after grafting. Asterisks indicate a significant difference between melon homo-grafted plants and hetero-grated plants using the Student's *t*-test ($p < 0.05$).

difference in graft compatible combinations (cucumber and pumpkin), while 12 of 27 graft incompatible combinations (luffa, wax gourd, bottle gourd, bitter gourd, and watermelon) had higher values when compared with melon homo-grafted plants (**Figure 4C**).

Starch Accumulation Above and Below the Graft Junction of Melon Grafted Onto Eight *Cucurbitaceae* Species

Starch–iodine staining can indicate the starch accumulation in plant tissue (Santacruz et al., 2005). According to the staining area, only around 10% area of melon scion stems was stained above 2 mm of the graft junction when grafted onto cucumber, pumpkin, and melon rootstocks on day 14 after grafting, while 27–70% area was stained when the melon was grafted onto luffa, wax gourd, bottle gourd, bitter gourd, and watermelon rootstocks (**Figure 5**). Only around 10% area of the rootstock stem was stained below 2 mm of the graft junction in all grafted plants on day 14 (**Supplementary Figure 4**). On day 42 after grafting, the staining tendency was similar as on day 14 after grafting (**Supplementary Figure 5**).

Correlation Analysis Between Measured Parameters of Melon Grafted Onto Eight *Cucurbitaceae* Species

Scion dry weight on day 42 after grafting was significantly correlated with plant height on day 42 ($r = 0.98^{***}$) and day 28 ($r = 0.83^{***}$), rootstock diameter on day 42 ($r = 0.80^{***}$), and scion starch staining ratio on day 14 ($r = -0.87^{***}$). Scion starch staining ratio was significantly correlated with plant height on day 28 ($r = -0.84^{***}$) and day 42 ($r = -0.88^{***}$) (**Figure 6**). However, other parameters on day 14, including scion height ($r = 0.66^{***}$), rootstock diameter ($r = 0.55^{***}$), stem diameter ratio ($r = -0.49^{**}$), leaf area ratio ($r = 0.36^{*}$), and SPAD ($r = -0.66^{***}$) were significantly correlated with scion dry weight on day 42 after grafting (**Figure 6**).

Anatomical Observation of the Grafted Junction

To observe the graft junction, one cultivar of each rootstock species was selected, using melon as the scion, and their anatomical analyses were performed on day 14 after grafting (**Figures 7**, **8**). We observed the completed newborn vascular bundle passing through the graft junction in all grafted combinations (**Figures 7**, **8**). However, more cell proliferation was observed at graft junctions in hetero-grafted plants when compared to homo-grafted plants. The compatible rootstocks cucumber cv. 'Jinyou No.35' and pumpkin cv. 'Qingyouzhen No.1' grafted combinations showed more cell proliferation than the incompatible rootstocks luffa cv. 'Sanbi No.6,' wax gourd cv. 'Aonong,' bottle gourd cv. 'H19,' bitter gourd cv. 'Liangku No.1,' and watermelon cv. 'Zaojia 8424' grafted combinations (**Figure 7**). We observed the undegraded necrotic layer fragments at the graft junction of bitter gourd and watermelon rootstocks, especially bitter gourd (**Figures 7F,G**). The aniline blue staining

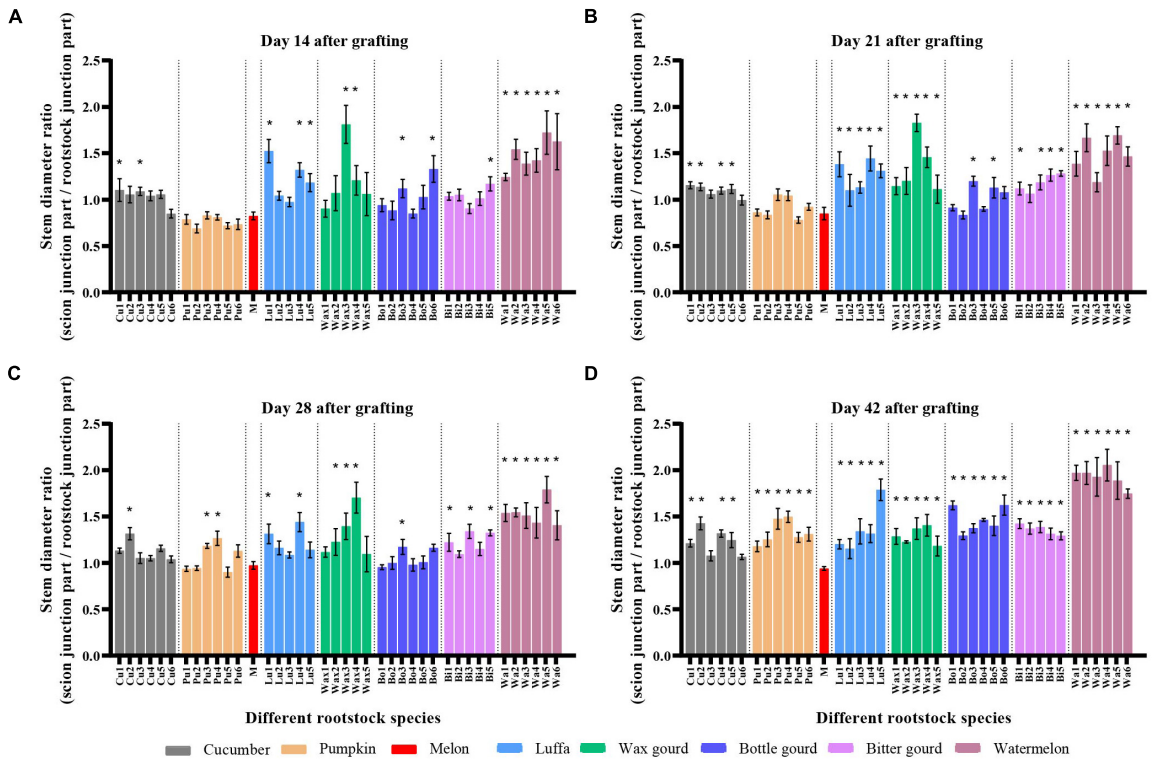

FIGURE 3 | The stem diameter ratio of scion and rootstock of melon grafted onto eight *Cucurbitaceae* rootstocks. The stem diameter ratio of scion and rootstock (above and below 5 mm of the graft junction) of melon cv. 'Akekekouqi' grafted onto cucumber (Cu1–Cu6), pumpkin (Pu1–Pu6), melon (M), luffa (Lu1–Lu5), wax gourd (Wax1–Wax5), bottle gourd (Bo1–Bo6), bitter gourd (Bi1–Bi5), and watermelon (Wa1–Wa6) on day 14 **(A)**, day 21 **(B)**, day 28 **(C)**, and day 42 **(D)** after grafting. Asterisks indicate a significant difference between melon homo-grafted plants and hetero-grated plants using the Student's *t*-test ($p < 0.05$).

results indicated that excess callose was deposited in new-vascular tissue in luffa, wax gourd, bottle gourd, bitter gourd, and watermelon rootstocks grafted combinations (**Figure 8**).

DISCUSSION

Melon Graft Compatibility in *Cucurbitaceae*

Previous studies on the evaluation of melon graft compatibility mainly focused on a small number of rootstock species, such as wild watermelon, pumpkin, and bottle gourd (Cohen et al., 2007; Aloni et al., 2008; Shang et al., 2016; Zhou et al., 2018; Camalle et al., 2021). In this study, 40 cultivars were selected to evaluate the melon graft compatibility, including eight species of *Cucurbitaceae* (cucumber, pumpkin, melon, luffa, wax gourd, bottle gourd, bitter gourd, and watermelon), which gave a more comprehensive evaluation on graft compatibilities between melon and a range of *Cucurbitaceae* species. Based on the melon scion dry weight on day 42 after grafting, it was found that cucumber and pumpkin were the graft compatible rootstocks with melon, while luffa, wax gourd, bottle gourd, bitter gourd, and watermelon were the graft incompatible rootstocks. Other studies showed that there was a difference in the compatibility of melon grafted onto different pumpkin cultivars (Aloni et al., 2008; Camalle et al., 2021). However, melon cv. 'Akekekouqi' was compatible when grafted onto all six pumpkin cultivars in this study; different scion and rootstock combinations could explain the discrepancy. Melon scion growth was normal when compatible rootstocks were used. However, the evaluation of incompatible rootstock combinations should be carefully done. For example, bitter gourd rootstock combinations showed higher scion height on day 14 and day 21, while on day 42, the scion height was significantly lower than that of the compatible rootstocks (**Figure 2**). Therefore, the graft compatibility evaluation of melon should be done over a relatively long time period if only considering plant growth parameters.

Starch–Iodine Staining Is an Index That Can Be Used to Evaluate the Melon Graft Compatibility Earlier

Graft incompatibility results in an inhibition of plant growth and physiological function, such as plant height suspension, swollen graft junction, and lower photosynthesis (Cohen et al., 2007; Kawaguchi et al., 2008; Xu et al., 2015; Chen Z. et al., 2016). According to our study, the scion height between incompatible and compatible combinations cannot be accurately distinguished until day 42 after grafting for all species. Rootstock stem diameter

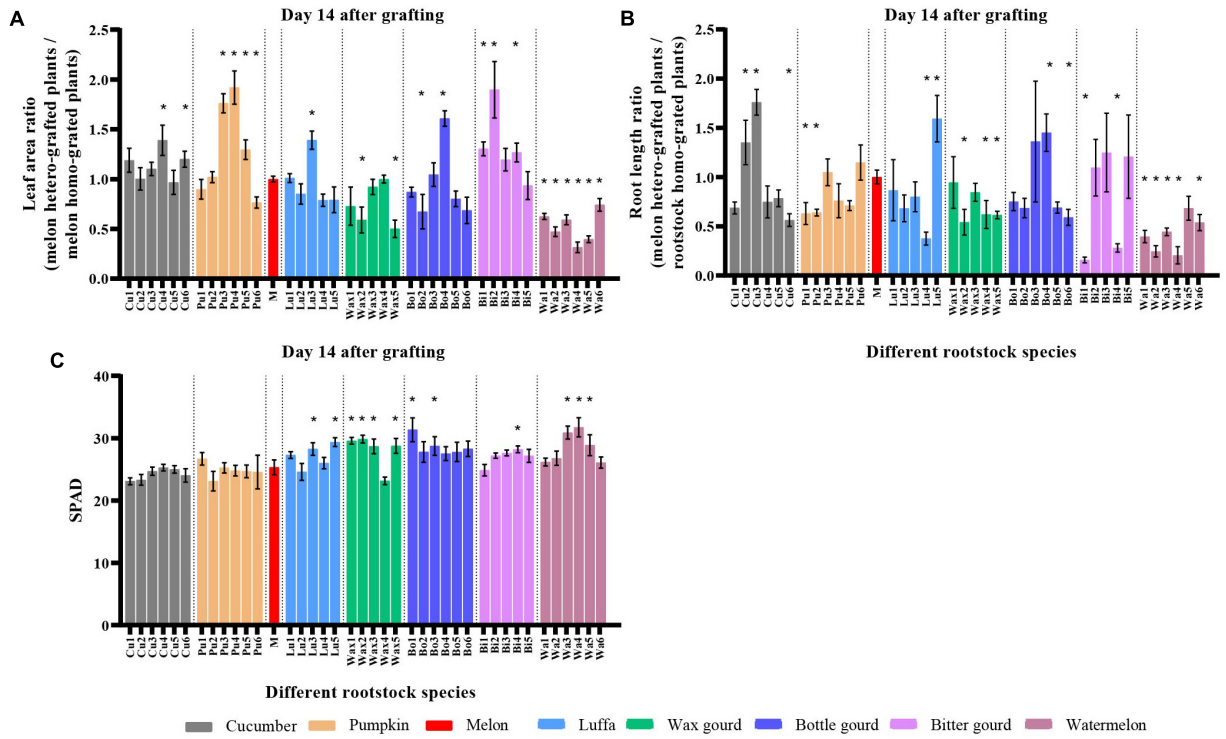

FIGURE 4 | Leaf area, root length, and SPAD of melon grafted onto eight *Cucurbitaceae* rootstocks on day 14 after grafting. Leaf area ratio (**A**, melon hetero-grafted plants/melon homo-grafted plants), root length ratio (**B**, melon hetero-grafted plants/rootstock homo-grafted plants), and SPAD (**C**, relative chlorophyll value) of melon cv. 'Akekekouqi' grafted onto cucumber (Cu1–Cu6), pumpkin (Pu1–Pu6), melon (M), luffa (Lu1–Lu5), wax gourd (Wax1–Wax5), bottle gourd (Bo1–Bo6), bitter gourd (Bi1–Bi5), and watermelon (Wa1–Wa6). Asterisks indicate a significant difference between melon or rootstock homo-grafted plants and hetero-grafted plants using Student's *t*-test ($p < 0.05$).

showed a decreased trend between compatible and incompatible combinations on day 42. Although the correlation heat map showed that plant height on day 42 and rootstock diameter on day 42 were significantly correlated with scion dry weight, they cannot be used as an index to evaluate the melon graft compatibility earlier than scion dry weight.

For the early physiological response on day 14 after grafting, we could not see lower values of SPAD (relative chlorophyll value) in all incompatible graft combinations, which was inconsistent with the values of Xu et al. (2015), who reported the decrease in chlorophyll fluorescence levels and chlorophyll contents on day 25 after grafting in incompatible cucumber graft combinations. Chen Z. et al. (2016) reported the decrease in net photosynthetic rate and chlorophyll contents after 6 months of litchi grafted onto incompatible rootstocks. We thought that in this study, more time was needed to observe the decrease in SPAD because the decrease in photosynthesis of incompatible graft cucumber was noticed on day 25 after grafting (Xu et al., 2015). In addition, other candidate indexes on day 14, such as leaf area ratio, root length ratio, scion diameter, scion height, rootstock diameter, stem diameter ratio, and rootstock starch staining ratio, were not strongly correlated with scion day weight on day 42 after grafting; the scion dry weight is a key parameter to evaluate melon graft compatibility. Therefore, the above indexes are not suitable to evaluate the melon graft compatibility at an earlier stage.

Sugar response ranged from asymmetric to symmetric was the main event in graft healing development (Melnyk et al., 2018). A previous study showed that there was an accumulation of sugars and sugar alcohols, such as glucose, fructose, galactose, and sucrose, in the scion of melon grafted onto incompatible rootstocks (Camalle et al., 2021). Meanwhile, when plants experience huge fluctuations in available carbon, such as that caused by a photosynthetic rate change, abiotic stress, or other unusual situations, they will accumulate and remobilize starch to slow down changes in the carbon balance (Gibon et al., 2009; Stitt et al., 2010). For example, increased sugar levels in leaves can activate the AGPase activity and promote the synthesis of starch during the day (Geigenberger et al., 2005). Starch, as a storage carbohydrate deposited in plant tissues, can be characterized using Iodine solution (Santacruz et al., 2005). Therefore, we tried to build the starch–iodine staining to evaluate the graft compatibility. Based on starch–iodine staining results, the starch accumulation was asymmetric between scion and rootstock in incompatible combinations, while symmetric starch accumulation in compatible combinations was observed on day 14 after grafting. Scion stem starch–iodine staining on day 14 was significantly correlated with scion dry weight on day 42 after grafting (**Figure 6**), suggesting that it was an index to evaluate the melon compatibility earlier, instead of long-time measurement of scion dry weight, height, and rootstock stem

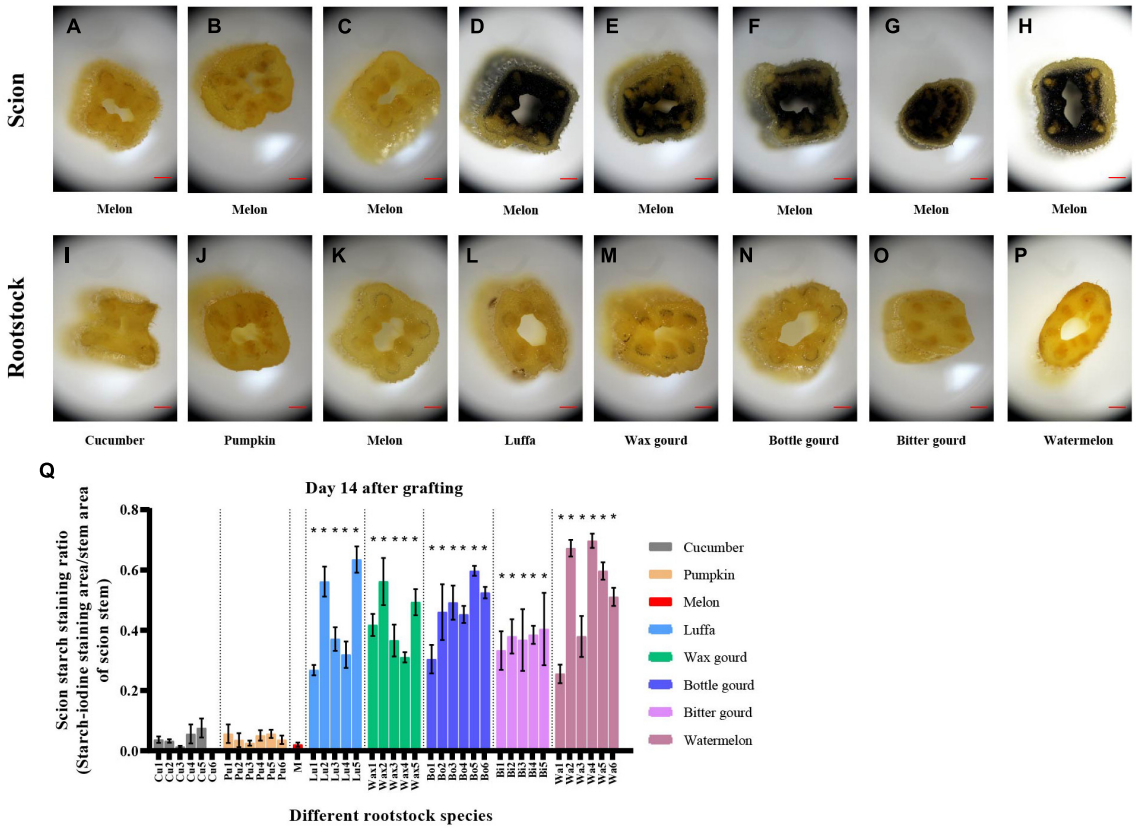

FIGURE 5 | Starch accumulations above and below the graft junction of melon grafted onto eight *Cucurbitaceae* rootstocks on day 14 after grafting. **(A–H)** Starch–iodine staining of scion stem above 2 mm of the graft junction of melon cv. 'Akekekouqi' grafted onto cucumber cv. 'Jinyou No.35,' pumpkin cv. 'Qingyouzhen No.1,' melon cv. 'Akekekouqi,' luffa cv. 'Sanbi No.6,' wax gourd cv. 'Aonong,' bottle gourd cv. 'H19,' bitter gourd cv. 'Liangku No.1,' and watermelon cv. 'Zaojia 8424.' Scale bar represents 500 μm. **(I–P)** Starch–iodine staining of rootstock stem below 2 mm of the graft junction of melon cv. 'Akekekouqi' grafted onto cucumber cv. 'Jinyou No.35,' pumpkin cv. 'Qingyouzhen No.1,' melon cv. 'Akekekouqi,' luffa cv. 'Sanbi No.6,' wax gourd cv. 'Aonong,' bottle gourd cv. 'H19,' bitter gourd cv. 'Liangku No.1,' and watermelon cv. 'Zaojia 8424.' Scale bar represents 500 μm. **(Q)** Scion starch staining ratio (starch–iodine staining area/transected area of scion stem) of melon cv. 'Akekekouqi' grafted onto cucumber (Cu1–Cu6), pumpkin (Pu1–Pu6), melon (M), luffa (Lu1–Lu5), wax gourd (Wax1–Wax5), bottle gourd (Bo1–Bo6), bitter gourd (Bi1–Bi5), and watermelon (Wa1–Wa6) on day 14 after grafting. Asterisks indicate a significant difference between melon homo-grafted plants and hetero-grafted plants using the Student's *t*-test ($p < 0.05$).

diameter. Therefore, in the future, it would be convenient and efficient to evaluate the graft compatibility of melon earlier (on day 14 after grafting) by a starch–iodine staining of scion stem base, and since this procedure can be done at the seedling stage, even when the plants are still in the plug tray, there is no doubt that the cultivation space, resources, labor input, and evaluation time will be saved.

Compared to plant growth, plant metabolism responds earlier to the internal changes and environmental stimulus (Chen L. et al., 2016; Chen et al., 2017; Zhang et al., 2019). In *de novo* root regeneration of *Arabidopsis*, jasmonic acid (JA) and indole-3-acetic acid (IAA) accumulated within 4 h, but new roots appeared at least 6 days later (Chen L. et al., 2016; Zhang et al., 2019). Litchi showed a significant difference in IAA content between graft compatible and incompatible combinations at 2 h after grafting, but a difference in plant growth was observed for more than 30 days (Chen et al., 2017). Compared to graft compatible combinations, incompatible combinations accumulated excess starch at graft junction, but not all showed significant lower value in plant height and SPAD on day 14 after grafting (**Figures 2**, **4**, **5**), indicating that graft incompatibility induced significant differences in starch metabolism, which emerged earlier than in plant growth. In incompatible combinations of luffa, wax gourd, bottle gourd, and watermelon, there were significant differences in scion starch staining ratio between some cultivars in individual species on day 14 after grafting (**Figure 5**), such as Lu2 and Lu5 compared to Lu1, which could be due to differences in metabolic levels among different cultivars, and this led to varied starch accumulation at an early stage of graft formation in incompatible combinations. Similar results were also observed in melon plants grafted onto different watermelon cultivars, such as higher starch staining ratio but similar plant height in Wa2 and Wa4 compared to Wa3. In conclusion, the difference in metabolic levels of plants due to incompatibility precedes the difference in plant growth, which facilitated us to predict graft incompatibility by metabolic differences earlier.

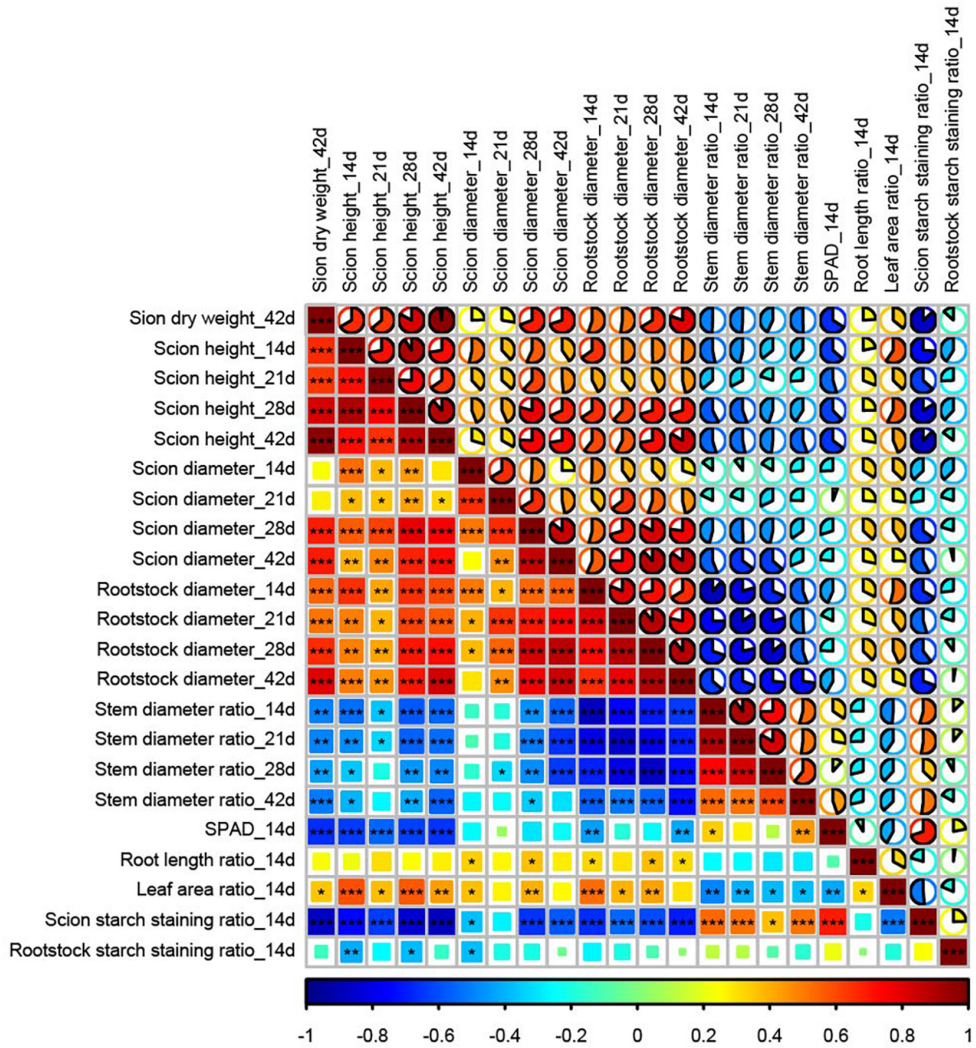

FIGURE 6 | Correlation analysis between parameters in melon grafted onto eight *Cucurbitaceae* rootstocks. Stem diameter ratio: stem diameter above 5 mm of the graft junction/stem diameter below 5 mm of the graft junction. Root length ratio: root length of melon hetero-grafted plants/root length of their rootstock homo-grafted plants. Leaf area ratio: scion leaf area of melon hetero-grafted plants/melon homo-grafted plants. Scion starch staining ratio: starch–iodine staining area/area of transected scion stem above 2 mm of the graft junction. Rootstock starch staining ratio: starch–iodine staining area/area of transected rootstock stem below 2 mm of the graft junction. Different colors, square size, and pie size indicate Pearson's correlation coefficient. Asterisks indicate significant correlation between two parameters (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Photoassimilate Transport Blockage and Undegraded Necrotic Layer Are Associated With Melon Incompatibility

The graft healing development is accompanied by cell proliferation, cell differentiation, and vascular reconnection at the graft junction (Melnyk et al., 2015). On day 14 after grafting, vascular reconnection events were observed in all grafted combinations. Meanwhile, more cell proliferation at graft junction was observed in hetero-grafted combinations, indicating that scion–rootstock combinations of different species could stimulate callus activities (**Figure 7**). Compatible combinations showed more cell proliferation than incompatible combinations in hetero-grafted plants, and undegraded necrotic layer fragments were observed at the graft junction of incompatible bitter gourd and watermelon rootstock combinations (**Figures 7F,G**). Therefore, although the vascular reconnection happened in incompatible combinations, the early graft healing process was still influenced by melon incompatibility, showing reduced callus activities and undegraded necrotic layer fragments at the graft junction, especially in incompatible bitter gourd and watermelon rootstock combinations (**Figure 7**).

As the photoassimilates, carbohydrates are synthesized from the leaves, transported to the whole body through the phloem sieve, and unloaded at the parenchyma cells through the symplast pathway in plants (Ma et al., 2019; Lu et al., 2020). As mentioned above, excess starch accumulated in the scion base

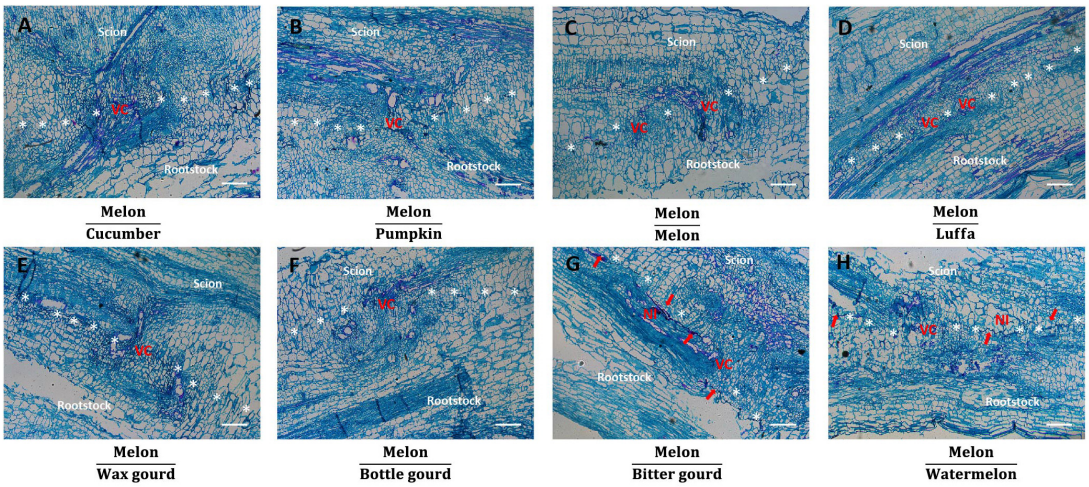

FIGURE 7 | Necrotic layer and vascular reconnection of melon grafted onto eight *Cucurbitaceae* species. **(A–H)** Longitudinal sections of the graft junction of melon cv. 'Akekekouqi' grafted onto cucumber cv. 'Jinyou No.35,' pumpkin cv. 'Qingyouzhen No.1,' melon cv. 'Akekekouqi,' luffa cv. 'Sanbi No.6,' wax gourd cv. 'Aonong,' bottle gourd cv. 'H19,' bitter gourd cv. 'Liangku No.1,' and watermelon cv. 'Zaojia 8424' on day 14 after grafting. Asterisks indicate graft junction. Red arrows indicate undegraded necrotic layer fragments. VC, vascular reconnection; NI, necrotic layer. Scale bar represents 250 μm.

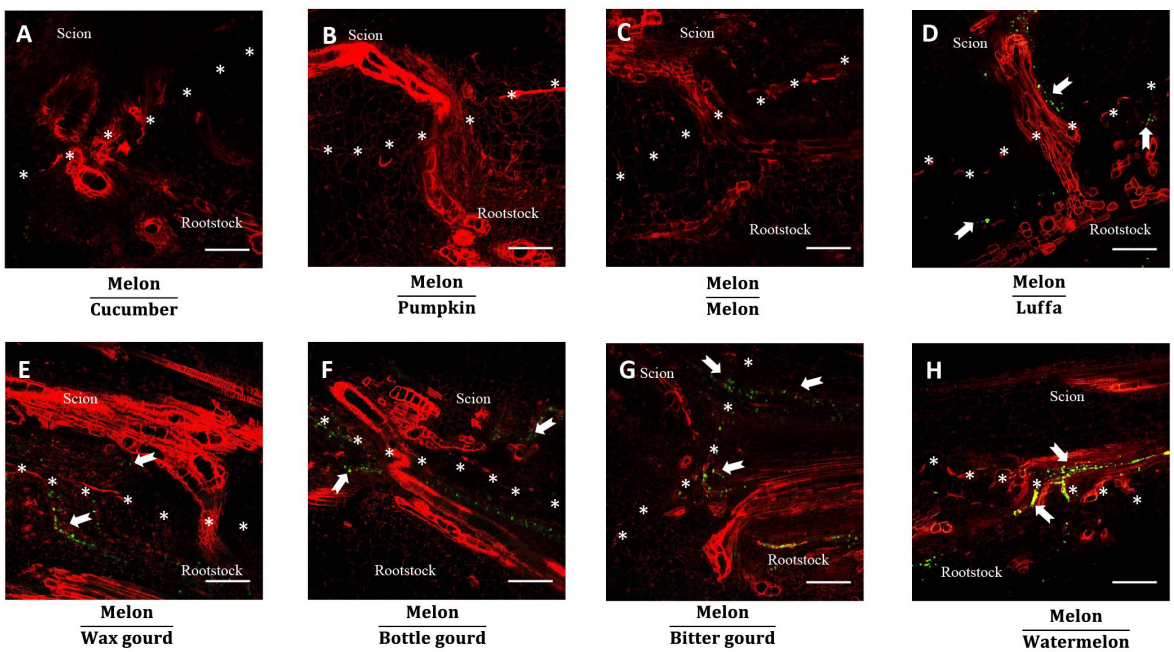

FIGURE 8 | Callose deposition of melon grafted onto eight *Cucurbitaceae* species. **(A–H)** Microscopic observation of the new vascular tissue at graft junction of melon cv. 'Akekekouqi' grafted onto cucumber cv. 'Jinyou No.35,' pumpkin cv. 'Qingyouzhen No.1,' melon cv. 'Akekekouqi,' luffa cv. 'Sanbi No.6,' wax gourd cv. 'Aonong,' bottle gourd cv. 'H19,' bitter gourd cv. 'Liangku No.1,' and watermelon cv. 'Zaojia 8424' on day 14 after grafting. Asterisks indicate graft junction. White arrows indicate callose deposition. Scale bar represents 200 μm.

of incompatible combinations, which indicated that there was a blockage of the transport of photoassimilates at the graft junction. These results are consistent with those findings obtained by Schoning and Kollmann (1997) and Espen et al. (2005), who used radiocarbon translocation and 5/6-carboxyfluorescein (CF) experiments to show the transport of photoassimilates *via* the phloem in incompatible grafted combinations. However, these studies only proposed the hypothesis of blockage based on transportation of photoassimilates and did not give detailed information about the blockage of phloem. In this study, microsection observation of fluorescent staining showed that excess callose was deposited in the newborn phloem

of incompatible combinations, while no callose deposition was observed in compatible combinations. Callose is mainly distributed in the phloem sieve and participates in regulating the organic transport of plants under unfavorable environments (Wu et al., 2018). Therefore, considering the inhibited plant growth of graft incompatible combinations, we think that callose deposition in new-born phloem, leading to the blockage of transport of photoassimilates, is the main reason for melon graft incompatibility. Further studies are needed to clarify the underlying mechanism of callose deposition in incompatible graft melons.

CONCLUSION

In this study, the compatibility of melon grafted onto eight *Cucurbitaceae* species including 40 cultivars was evaluated. Cucumber and pumpkin are graft compatible with melon, while luffa, wax gourd, bottle gourd, bitter gourd, and watermelon are graft incompatible with melon. Callose deposition and undegraded necrotic layer fragments at graft junction were the main reason for melon graft incompatibility. In this study, we proved that graft compatibility can be evaluated earlier (on day 14 after grafting) by a starch–iodine staining. This study provides clues for compatible melon rootstock selection and breeding.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MX, YH, and ZB devised the project. MX, LG, CL, JW, XW, and LL performed the experimental analyses. MX, CL, and YH performed the data analyses. MX and YH wrote the manuscript. All authors approved the final manuscript.

FUNDING

This research was funded by the National Natural Science Foundation of China (32172651), the National Key Research and Development Program of China (2019YFD1001900), China Agriculture Research System of MOF and MORA (CARS-25), and Hubei Provincial Natural Science Foundation of China (2019CFA017).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance and advice from Xiaoyang Wei (The University of Newcastle, Australia) in vibratome sectioning and fluorescence staining microscopic observations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.762889/full#supplementary-material

REFERENCES

Aloni, B., Karni, L., Deventurero, G., Levin, Z., Cohen, R., Katzir, N., et al. (2008). Possible mechanisms for graft incompatibility between melon scions and pumpkin rootstocks. *Acta Hortic.* 782, 313–324. doi: 10.17660/ActaHortic.2008.782.39

Camalle, M. D., Sikron, N., Zurgil, U., Khadka, J., Pivonia, S., Pěnčík, A., et al. (2021). Does scion-rootstock compatibility modulate photoassimilate and hormone trafficking through the graft junction in melon-pumpkin graft combinations? *Plant Sci.* 306:110852. doi: 10.1016/j.plantsci.2021.110852

Ceballos, H., Jaramillo, J., Salazar, S., Pineda, L., Calle, F., and Setter, T. (2017). Induction of flowering in cassava through grafting. *J. Plant Breed. Crop Sci.* 9, 19–29. doi: 10.5897/JPBCS2016.0617

Chen, L., Tong, J., Xiao, L., Ruan, Y., Liu, J., Zeng, M., et al. (2016). YUCCA-mediated auxin biogenesis is required for cell fate transition occurring during *de novo* root organogenesis in *Arabidopsis*. *J. Exp. Bot.* 67, 4273–4284. doi: 10.1093/jxb/erw213

Chen, Z., Zhao, J. T., Hu, F. C., Qin, Y. H., Wang, X. H., and Hu, G. B. (2017). Transcriptome changes between compatible and incompatible graft combination of *Litchi chinensis* by digital gene expression profile. *Sci. Rep.* 7:3954. doi: 10.1038/s41598-017-04328-x

Chen, Z., Zhao, J. T., Qin, Y. H., and Hu, G. B. (2016). Study on the graft compatibility between 'Jingganghongnuo' and other litchi cultivars. *Sci. Hortic.* 199, 56–62. doi: 10.1016/j.scienta.2015.12.020

Cohen, R., Burger, Y., Horev, C., Koren, A., and Edelstein, M. (2007). Introducing grafted cucurbits to modern agriculture - The Israeli experience. *Plant Dis.* 91, 916–923. doi: 10.1094/PDIS-91-8-0916

El-Gazzar, T. M., Dawa, K. K., Ibrahim, E. A., El-Banna, M., and Mohamed, A. (2017). Anatomical study onwatermelon grafting. *J. Plant Prod.* 8, 999–1009. doi: 10.21608/jpp.2017.41060

Espen, L., Cocucci, M., and Sacchi, G. A. (2005). Differentiation and functional connection of vascular elements in compatible and incompatible pear/quince internode micrografts. *Tree Physiol.* 25, 1419–1425. doi: 10.1093/treephys/25.11.1419

Flaishman, M. A., Loginovsky, K., Golobowich, S., and Lev-Yadun, S. (2008). *Arabidopsis thaliana* as a model system for graft union development in homografts and heterografts. *J. Plant Growth Regul.* 27, 231–239. doi: 10.1007/s00344-008-9050-y

Gautier, A. T., Chambaud, C., Brocard, L., Ollat, N., Gambetta, G. A., Delrot, S., et al. (2019). Merging genotypes: graft union formation and scion-rootstock interactions. *J. Exp. Bot.* 70, 747–755. doi: 10.1093/jxb/ery422

Geigenberger, P., Kolbe, A., and Tiessen, A. (2005). Redox regulation of carbon storage and partitioning in response to light and sugars. *J. Exp. Bot.* 56, 1469–1479. doi: 10.1093/jxb/eri178

Gibon, Y., Pyl, E. T., Sulpice, R., Lunn, J. E., Höhne, M., Günther, M., et al. (2009). Adjustment of growth, starch turnover, protein content and central metabolism to a decrease of the carbon supply when *Arabidopsis* is grown in very short photoperiods. *Plant. Cell. Environ.* 32, 859–874. doi: 10.1111/j.1365-3040.2009.01965.x

Gong, Y., Qi, Y., Fu, X., Yang, R., Chen, T., Tang, H., et al. (2016). Graft compatibility evaluation of different rootstock-scion combinations of honey poomelo at seedling stage. *J. Trop. Sub. Bot.* 24, 287–295.

Guo, J., Xu, W., Hu, Y., Huang, J., Zhao, Y., Zhang, L., et al. (2020). Phylotranscriptomics in *Cucurbitaceae* reveal multiple whole-genome

duplications and key morphological and molecular innovations. *Mol. Plant* 13, 1117–1133. doi: 10.1016/j.molp.2020.05.011

Hassell, R. L., Memmott, F., and Liere, D. G. (2008). Grafting methods for watermelon production. *Hortscience* 43, 1677–1679. doi: 10.21273/HORTSCI.43.6.1677

Huang, Y., Bie, Z. L., Liu, Z. X., Zhen, A., and Jiao, X. R. (2011). Improving cucumber photosynthetic capacity under NaCl stress by grafting onto two salt-tolerant pumpkin rootstocks. *Biol. Plant* 55, 285–290. doi: 10.1007/s10535-011-0040-8

Kawaguchi, M., Taji, A., Backhouse, D., and Oda, M. (2008). Anatomy and physiology of graft incompatibility in solanaceous plants. *J. Hortic. Sci. Biotechnol.* 83, 581–588. doi: 10.1080/14620316.2008.11512427

Lee, J. M., Kubota, C., Tsao, S. J., Bie, Z., Echevarria, P. H., Morra, L., et al. (2010). Current status of vegetable grafting: diffusion, grafting techniques, automation. *Sci. Hortic.* 127, 93–105. doi: 10.1016/j.scienta.2010.08.003

Liu, C., Lin, W., Feng, C., Wu, X., Fu, X., Xiong, M., et al. (2021). A new grafting method for watermelon to inhibit rootstock regrowth and enhance scion growth. [Preprint]. doi: 10.20944/preprints202107.0693.v1

Louws, F. J., Rivard, C. L., and Kubota, C. (2010). Grafting fruiting vegetables to manage soilborne pathogens, foliar pathogens, arthropods and weeds. *Sci. Hortic.* 127, 127–146. doi: 10.1016/j.scienta.2010.09.023

Lu, X. H., Liu, W. Q., Wang, T., Zhang, J. L., Li, X. J., and Zhang, W. N. (2020). Systemic long-distance signaling and communication between rootstock and scion in grafted vegetables. *Front. Plant Sci.* 11:460. doi: 10.3389/fpls.2020.00460

Ma, S., Sun, L. L., Sui, X. L., Li, Y. X., Chang, Y., Fan, J. W., et al. (2019). Phloem loading in cucumber: combined symplastic and apoplastic strategies. *Plant J.* 98, 391–404. doi: 10.1111/tpj.14224

Masayuki, O., Kunihiko, O., and Hidekazu, S. (2000). Effects of transplant container and *Solanum* rootstocks on the incidences of overgrowth and unmarketable fruits in tomato plants planted with plug seedlings. *Environ. Control Biol.* 38, 273–280. doi: 10.2525/ecb1963.38.273

Melnyk, C. W. (2017). Plant grafting: insights into tissue regeneration. *Regeneration* 4, 3–14. doi: 10.1002/reg2.71

Melnyk, C. W., and Meyerowitz, E. M. (2015). Plant grafting. *Curr. Biol.* 25, R183–R188. doi: 10.1016/j.cub.2015.01.029

Melnyk, C. W., Gabel, A., Hardcastle, T. J., Robinson, S., Miyashima, S., Grosse, I., et al. (2018). Transcriptome dynamics at *Arabidopsis* graft junctions reveal an intertissue recognition mechanism that activates vascular regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 115, E2447–E2456. doi: 10.1073/pnas.1718263115

Melnyk, C. W., Schuster, C., Leyser, O., and Meyerowitz, E. M. (2015). A developmental framework for graft formation and vascular reconnection in *Arabidopsis thaliana*. *Curr. Biol.* 25, 1306–1318. doi: 10.1016/j.cub.2015.03.032

Migicovsky, Z., Harris, Z. N., Klein, L. L., Li, M., McDermaid, A., Chitwood, D. H., et al. (2019). Rootstock effects on scion phenotypes in a 'Chambourcin' experimental vineyard. *Hort. Res.* 6:64. doi: 10.1038/s41438-019-0146-2

Nawaz, M. A., Imtiaz, M., Kong, Q. S., Cheng, F., Ahmed, W., Huang, Y., et al. (2016). Grafting: atechnique to modify ion accumulation in horticultural crops. *Front. Plant Sci.* 7:1457. doi: 10.3389/fpls.2016.01457

Nawaz, M. A., Wang, L. M., Jiao, Y. Y., Chen, C., Zhao, L., Mei, M. J., et al. (2017). Pumpkin rootstock improves nitrogen use efficiency of watermelon scion by enhancing nutrient uptake, cytokinin content, and expression of nitrate reductase genes. *Plant Growth Regul.* 82, 233–246. doi: 10.1007/s10725-017-0254-7

Niu, M. L., Huang, Y., Sun, S. T., Sun, J. Y., Cao, H. S., Shabala, S., et al. (2018). Root respiratory burst oxidase homologue-dependent H_2O_2 production confers salt tolerance on a grafted cucumber by controlling Na^+ exclusion and stomatal closure. *J. Exp. Bot.* 69, 3465–3476. doi: 10.1093/jxb/erx386

Notaguchi, M., Kurotani, K., Sato, Y., Tabata, R., Kawakatsu, Y., Okayasu, K., et al. (2020). Cell-cell adhesion in plant grafting is facilitated by beta-1,4-glucanases. *Science* 369, 698–702. doi: 10.1126/science.abc3710

Ren, Y., Guo, S. R., Shu, S., Xu, Y., and Sun, J. (2018). Isolation and expression pattern analysis of *CmRNF5* and *CmNPH3L* potentially involved in graft compatibility in cucumber/pumpkin graft combinations. *Sci. Hortic.* 227, 92–101. doi: 10.1016/j.scienta.2017.09.022

Santacruz, S., Andersson, R., and Aman, P. (2005). Characterisation of potato leaf starch with iodine-staining. *Carbohydr. Polym.* 59, 397–400. doi: 10.1016/j.carbpol.2004.10.001

Schoning, U., and Kollmann, R. (1997). Phloem translocation in regenerating in vitro heterografts of different compatibility. *J. Exp. Bot.* 48, 289–295. doi: 10.1093/jxb/48.2.289

Shang, J., Wang, J., Li, N., and Ma, S. (2016). Research on the compatibility of grafted melon of different stocks. *China Cucurbits Veg.* 29, 38–40.

Souza, L. S., Diniz, R. P., Neves, R. D. J., Alves, A. A. C., and Oliveira, E. J. (2018). Grafting as a strategy to increase flowering of cassava. *Sci. Hortic.* 240, 544–551. doi: 10.1016/j.scienta.2018.06.070

Stitt, M., Lunn, J., and Usadel, B. (2010). Arabidopsis and primary photosynthetic metabolism - more than the icing on the cake. *Plant J.* 61, 1067–1091. doi: 10.1111/j.1365-313X.2010.04142.x

Sun, X., Pan, X., Wang, Y., Bai, Y., Zong, C., Qi, Y., et al. (2015). Anatomical study of integration sections for distant grafting between soybean and pumpkin. *Soyb. Sci.* 34, 639–642.

Thomas, H., Broeck, L. V., Spurney, R., Sozzani, R., and Frank, M. (2021). *Gene Regulatory Networks for Compatible Versus Incompatible Grafts Identify a Role for SlWOX4 During Junction Formation. bioRxiv* [Preprint]. Available online at: https://doi.org/10.1101/2021.02.26.433082 (accessed February 27, 2021).

Wang, L. K., Li, J. J., Jiang, W., Huang, H. L., Zheng, L. L., and Zhao, Y. (2018). *Vibrating Section. Bio-101: e1010138*. Available online at: https://doi.org/10.21769/BioProtoc.1010138. (accessed April 27, 2018).

Wu, S. W., Kumar, R., Iswanto, A. B. B., and Kim, J. Y. (2018). Callose balancing at plasmodesmata. *J. Exp. Bot.* 69, 5325–5339. doi: 10.1093/jxb/ery317

Xiang, G., Shao, X., and Yang, S. (1992). Comparative observation on cytology during the formation of a compatible autograft and an incompatible heterograft. *Acta Agric. Univ. Pekinensis* 18, 267–273.

Xu, Q., Guo, S. R., Li, L., An, Y. H., Shu, S., and Sun, J. (2016). Proteomics analysis of compatibility and incompatibility in grafted cucumber seedlings. *Plant Physiol. Biochem.* 105, 21–28. doi: 10.1016/j.plaphy.2016.04.001

Xu, Q., Guo, S. R., Li, L., Du, N. S., and Shu, S. (2015). Physiological aspects of compatibility and incompatibility in grafted cucumber seedlings. *J. Amer. Soc. Hortic. Sci.* 140, 299–307. doi: 10.21273/JASHS.140.4.299

Yetisir, H., and Sari, N. (2003). Effect of different rootstock on plant growth, yield and quality of watermelon. *Aust. J. Exp. Agric.* 43, 1269–1274. doi: 10.1071/EA02095

Yetisir, H., Kurt, S., Sari, N., and Tok, F. M. (2007). Rootstock potential of Turkish *Lagenaria siceraria* germplasm for watermelon: plant growth, graft compatibility, and resistance to *Fusarium*. *Turk J. Agric. For.* 31, 381–388.

Zhang, G., Zhao, F., Chen, L., Pan, Y., Sun, L., Bao, N., et al. (2019). Jasmonate-mediated wound signalling promotes plant regeneration. *Nat. Plants* 5, 491–497. doi: 10.1038/s41477-019-0408-x

Zhong, Y. Q., Chen, C., Nawaz, M. A., Jiao, Y. Y., Zheng, Z. H., Shi, X. F., et al. (2018). Using rootstock to increase watermelon fruit yield and quality at low potassium supply: a comprehensive analysis from agronomic, physiological and transcriptional perspective. *Sci. Hortic.* 241, 144–151.

Zhou, J., Liu, W., Zhang, M., Fan, A., Chen, B., Lu, Z., et al. (2018). Effects of symbiosis incompatibility rootstock on growth and physiology indicators of grafted melon. *Southwest China J. Agric. Sci.* 31, 1606–1610.

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

REVIEW
published: 04 November 2021
doi: 10.3389/fpls.2021.741009

SuperSour: A New Strategy for Breeding Superior Citrus Rootstocks

Kim D. Bowman[1], Greg McCollum[1] and Ute Albrecht[2]*

[1] U.S. Horticultural Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Ft. Pierce, FL, United States, [2] Southwest Florida Research and Education Center, Horticultural Sciences Department, Institute of Food and Agricultural Sciences, University of Florida, Immokalee, FL, United States

Citrus crops have a long history of cultivation as grafted trees on selected rootstock cultivars, but all current rootstocks have significant limitations and traditional methods of rootstock breeding take at least 2–3 decades to develop and field test new rootstocks. Citrus production in the United States, and other parts of the world, is impaired by a wide range of biotic and abiotic problems, with especially severe damage caused by the disease huanglongbing (HLB) associated with *Candidatus* Liberibacter asiaticus. All major commercial citrus scion cultivars are damaged by HLB, but tree tolerance is significantly improved by some rootstocks. To overcome these challenges, the USDA citrus breeding program has implemented a multi-pronged strategy for rootstock breeding that expands the diversity of germplasm utilized in rootstock breeding, significantly increases the number of new hybrids evaluated concurrently, and greatly reduces the time from cross to potential cultivar release. We describe the key components and methodologies of this new strategy, termed "SuperSour," along with reference to the historical favorite rootstock sour orange (*Citrus aurantium*), and previous methods employed in citrus rootstock breeding. Rootstock propagation by cuttings and tissue culture is one key to the new strategy, and by avoiding the need for nucellar seeds, eliminates the 6- to 15-year delay in testing while waiting for new hybrids to fruit. In addition, avoiding selection of parents and progeny based on nucellar polyembryony vastly expands the potential genepool for use in rootstock improvement. Fifteen new field trials with more than 350 new hybrid rootstocks have been established under the SuperSour strategy in the last 8 years. Detailed multi-year performance data from the trials will be used to identify superior rootstocks for commercial release, and to map important traits and develop molecular markers for the next generation of rootstock development. Results from two of these multi-year replicated field trials with sweet orange scion are presented to illustrate performance of 97 new hybrid rootstocks relative to four commercial rootstocks. Through the first 7 years in the field with endemic HLB, many of the new SuperSour hybrid rootstocks exhibit greatly superior fruit yield, yield efficiency, canopy health, and fruit quality, as compared with the standard rootstocks included in the trials.

Keywords: fruit yield, genetic mapping, citrus rootstock, breeding strategy, huanglongbing disease

OPEN ACCESS

Edited by:
Sergio Ruffo Roberto, State University of Londrina, Brazil

Reviewed by:
Eduardo Augusto Girardi, Brazilian Agricultural Research Corporation (EMBRAPA), Brazil
Mikeal Levi Roose, University of California, Riverside, United States
Fernando Alves De Azevedo, Sylvio Moreira Citriculture Center, Brazil

****Correspondence:***
Kim D. Bowman
kim.bowman@usda.gov

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *14 July 2021*
Accepted: *14 October 2021*
Published: *04 November 2021*

Citation:
Bowman KD, McCollum G and Albrecht U (2021) SuperSour: A New Strategy for Breeding Superior Citrus Rootstocks. Front. Plant Sci. 12:741009. doi: 10.3389/fpls.2021.741009

INTRODUCTION

The genus *Citrus* encompasses a broad range of fruit crops of worldwide importance, including sweet orange (*Citrus sinensis* [L.] Osbeck), mandarin (*C. reticulata* Blanco), grapefruit (*C. paradisi* Macf.), and lemon (*C. limon* [L.] Burm. f.), all of which are usually grown in modern citriculture as grafted trees on selected specialized rootstock cultivars. Although most selection of citrus in early history focused on fruit and above-ground tree characteristics, many natural citrus selections with useful rootstock characteristics were identified before the beginning of modern directed citrus breeding (Bitters et al., 1969). These natural selections provided the major rootstocks employed during the early use of grafted citrus trees in the 19th century. Directed citrus breeding for improved scions began in the late 19th century (Cooper et al., 1962), and related interest in breeding for improved rootstocks began shortly afterward. As a result of selection among natural variants and directed breeding efforts, there are now hundreds of different rootstock clones with some commercial use in the many countries involved in citrus production. In Florida, more than 40 different rootstocks are used for propagation in commercial citrus nurseries each year (Rosson, 2020), as growers attempt to find the best rootstock for each situation. Unfortunately, all currently used rootstocks have known or indeterminate faults (Bowman and Joubert, 2020), which limit their survival, tolerance of disease, adaptability, or positive influence on fruit productivity or quality.

In particular, threats from a range of common diseases and pests have continued to reduce tree health and cause major losses in terms of tree survival and productivity in most production regions. The disease huanglongbing (Bove, 2006), associated with *Candidatus* Liberibacter asiaticus (CLas) and other *Ca.* Liberibacter species, is widespread in the Eastern Hemisphere and has spread to most of the citrus production area in the Western Hemisphere over the last 15 years. In Florida and Brazil, the disease is causing devastating declines in productivity (Alvarez et al., 2016; Bassanezi et al., 2020; Li et al., 2020) and fruit quality (Dala-Paula et al., 2019). Perhaps of the greatest commercial significance, all existing sweet orange and grapefruit cultivars and many mandarins are highly susceptible and severely affected by huanglongbing (HLB) disease. While specialized intensive management practices can somewhat slow disease spread and lessen the disease effects (Stansly et al., 2014), these practices have limited effectiveness and incur significant costs that often eliminate the profitability of growing the citrus crop. In addition, many of the management practices required to effectively moderate disease spread and limit decline in production involve intensive use of pesticides and nutritional treatments that threaten the environment. As a partial solution, good tolerance to CLas infection has been demonstrated in citrus germplasm, and some existing rootstocks such as US-942, have demonstrated field tolerance to HLB that can significantly improve health and productivity of sweet orange trees in an HLB-endemic environment (Bowman and McCollum, 2015; Bowman et al., 2016a,b). However, even with the use of the best of the currently available HLB-tolerant rootstocks, sweet orange and grapefruit trees suffer a significantly reduced tree health, and loss in fruit production and fruit quality, as compared to non-infected trees. Improved citrus rootstocks are urgently needed to provide better tolerance or resistance to diseases such as HLB, as well as improved influence on fruit production and fruit quality of the scion. While traditional citrus breeding efforts by USDA and others have been historically successful in producing new rootstocks of commercial value (Bowman and Joubert, 2020), the pace of these efforts has been very slow, taking several decades from cross to commercial release. In addition, previous conventional citrus rootstock breeding by USDA and several other programs focused on relatively limited portions of the citrus genepool because of an obligatory apomictic seed reproduction that has characterized essentially all citrus rootstocks in commercial use. In this article, we describe a new multi-pronged strategy for citrus rootstock breeding in the USDA program, which includes greater genetic diversity and expanded concurrent early-stage testing of a large number of new hybrids. The strategy significantly reduces the time from cross to release of a new hybrid rootstock by eliminating apomictic seed reproduction as a prerequisite for field-testing and as a requirement for new rootstocks. Although uniform seed propagation is convenient and economical for the citrus nursery, the historical focus on nucellar polyembryony as a primary rootstock trait has become a critical limitation in traditional citrus rootstock breeding. The expanded concurrent and replicated testing of hundreds of new hybrid rootstocks also is providing extensive genotype-performance information that will be used to map important genetic traits and develop molecular markers to power the next generation of citrus rootstock breeding.

HISTORY OF CITRUS ROOTSTOCKS AND BREEDING

Early cultivation of citrus crops undoubtedly relied on seedling trees chosen for their favorable fruit quality traits. Some citrus species and selections have a reproductive trait known as nucellar polyembryony (Frost and Soost, 1968; Koltunow et al., 1995; Kepiro and Roose, 2007), which causes most seedlings to be derived by apomixis from maternal tissue and genetically identical to the seed source tree. In citrus, the apomictic production of seedlings for many species and selections made rapid and uniform seed propagation of many fruiting types quite easy, and probably had an important role in early selection of those types that were easy to propagate by seed. The existence of nucellar polyembryony, and resulting easy uniform propagation by seed, in sweet orange, sour orange (*Citrus aurantium* L.), and lemon was probably a critical reason for the widespread distribution of these types in early history, as seedlings grown for their fruit.

There is mention of grafting citrus in historical accounts in Chinese, Greek, and Roman literature from 1000 to 2000 years ago (Bitters, 1986), but it appears that this was usually as a curiosity for citrus in botanical collections, arboretums, and orangeries (Webber, 1967), rather than a practice for widespread fruit production. Undoubtedly, the ease of propagating many

citrus fruiting varieties with good fidelity through nucellar seedlings reduced the motivation for grafting and rootstock use in these crops. Historical accounts suggest the first significant use of grafted trees for large-scale citrus production began about the mid- to late 1800s in the United States, and was driven primarily by *Phytophthora* disease problems on seedling trees in Florida (Castle, 2010), and the desire to vegetatively propagate specific clones of seedless sweet orange (such as Washington Navel orange) in California (Bitters, 1986). Initially, the use of grafting in citrus commercial production appears to have been as topworking of existing seedling trees growing in the field, rather than production in a tree nursery. The topworking of wild sour orange groves with sweet orange in Florida began as early as 1830, but was very successful and became widespread by the late 1800s (Webber, 1967). Through the end of the 19th century, all citrus varieties in existence were natural species or the result of natural hybridizations, and any efforts at using citrus rootstocks relied upon those naturally occurring varieties.

The rootstock with greatest fame and notoriety may be sour orange, or the genetic clone that has long been classified as *Citrus aurantium*. Sour orange was originally selected for cultivation because of its fragrance and fruit that was valued for flavoring fish and meat (Tolkowsky, 1938), rather than as a fresh fruit or a rootstock. Over time, interest in sour orange as a fruiting variety declined, but it came to be recognized as possessing many excellent rootstock traits, including uniform seed propagation, broad soil adaptability, and good influence on fruit cropping and fruit quality of a grafted scion. Sour orange was probably the most often used rootstock during the early era of grafted citrus trees in most citrus regions. In Florida, established groves of sour orange seedlings were topworked with sweet orange during the mid-1800s with great success, and contributed to the rapid expansion of the citrus industry in Florida (Webber, 1967). However, sour orange also has a flaw in its sensitivity to citrus tristeza virus (CTV) when used as a rootstock with most scions, and there are numerous examples of disastrous citrus industry losses because of CTV-induced quick decline in trees on this rootstock. Historically, major tree losses from CTV infection of citrus trees on sour orange rootstock have been reported in Argentina, Brazil, Ghana, and California (Bar-Joseph et al., 1989). Despite this risk, sour orange still has some popularity in many citrus growing areas and is regarded by many as one of the best citrus rootstocks (except for the Achilles heel of CTV sensitivity) because of its good adaptability to unfavorable soil conditions and good influence on fruit quality. One of the citrus production areas where sour orange is still regarded very highly is the Indian River Region of Florida, where it is especially considered as an outstanding rootstock for grapefruit (Castle et al., 2011). Although sour orange is given species status in botanical classification, it is now identified from several different lines of evidence, including nuclear (Wu et al., 2014) and chloroplast (Carbonell-Caballero et al., 2015) sequencing studies, to be derived from a single natural hybridization of pummelo (*Citrus maxima* [L.] Osbeck) as the seed parent, and mandarin (*Citrus reticulata*) as the pollen parent. That natural hybridization probably occurred about 3,000 years ago (Carbonell-Caballero et al., 2015). Over the last 30 centuries, point mutations have created a multitude of sour orange variants with similar fruit and horticultural traits (Bowman and Garnsey, 2001), and the many small variations on this interspecific hybrid have been spread widely through genetically identical apomictic seedlings produced by nucellar polyembryony.

The clearest evidence of a transition to a more scientific approach to breeding new citrus varieties was in the establishment of the USDA Subtropical Laboratory at Eustis, Florida, in 1892, where H. J. Webber and W. T. Swingle began research on citrus diseases and breeding. Over the following 30 years, similar efforts to begin scientific research directed at solving citrus problems, in part through breeding, began at University of California, University of Florida, and in Europe, South Africa, and Japan (Webber, 1967). The earliest focus of breeding in the USDA program in Florida was to obtain fruiting varieties with greater cold hardiness, a response to the disastrous freeze in the winter of 1894/1895. This effort, which focused most heavily on crossing the cold hardy citrus relative trifoliate orange (*Poncirus trifoliata* [L.] Raf.) with sweet orange and grapefruit, was not successful in producing cold hardy scion varieties because of very acrid fruit flavors found in first- and second-generation hybrids of citrus with trifoliate orange. However, out of this effort a series of trifoliate orange hybrids were produced which eventually became recognized as having good rootstock traits. Two of these trifoliate hybrids, Carrizo citrange (sweet orange × trifoliate orange) and Swingle citrumelo (grapefruit × trifoliate orange) were eventually released by USDA as rootstocks, in 1934 and 1974 (Hutchison, 1974; Wutscher, 1979), respectively, and have become major rootstocks with commercial use in all regions of the world (Bowman and Joubert, 2020). The USDA citrus rootstock breeding program was revived beginning in 1992, and the program released several other trifoliate hybrids that became important in Florida production during 2001–2020 (Bowman et al., 2016a). Testing of candidate hybrid rootstocks under the USDA breeding program has included focused evaluation of selected traits of horticultural importance, including nursery traits (Bowman et al., 1995, 1997, 2016a; Barcelos-Bisi et al., 2020) and tolerance to diseases and pests (Bowman and Garnsey, 2001; Bowman et al., 2001, 2002, 2003; Albrecht and Bowman, 2004, 2007, 2011, 2012, 2019; Graham et al., 2007; Xiang et al., 2010; Bowman and Albrecht, 2015, 2020), but focus of evaluation and selection was on field performance under typical commercial production conditions. Similar rootstock breeding efforts to cross trifoliate orange with sweet orange, grapefruit, and mandarin were also conducted at University of Florida (Castle, 1987; Castle et al., 1988) and University of California (Bitters et al., 1964; Cameron and Soost, 1986), as well as by researchers in Europe (Forner et al., 2003), South Africa (von Broembsen, 1985; Lee et al., 2009), and Australia (Long et al., 1977; Freeman et al., 1986). Other than work with the USDA, recent rootstock breeding efforts are best documented for programs at University of Florida (United States), Valencian Institute of Agrarian Research (IVIA, Spain), Council for Agricultural Research and Economics (CREA, Italy), and Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Brazil).

At University of Florida, active work to breed new citrus rootstocks has been underway by a team at Lake Alfred for more than 35 years. Much of the early work by the team made use of somatic hybridization, and then subsequent crosses among allotetraploid products of that prior work (Grosser et al., 2004; Grosser and Gmitter, 2011). Sexual hybridization among diploid parents was also used in the UF program to develop new rootstock hybrids. During the evolution of this program, the UF team began to use "the gauntlet" method for screening new rootstock hybrids, which involves the screening of individual hybrid seedlings sequentially by high pH, *Phytophthora* infestation, and HLB exposure to identify those most hardy to survive these stresses, followed by a field trial with the survivors (Grosser et al., 2016, 2020). Nine new UF citrus rootstocks were released in 2013 (UFR-1, UFR-2, UFR-3, UFR-4, UFR-5, UFR-6, UFR-15, UFR-16, and UFR-17)[1]. The most popular of those is UFR-4, which was used for 49,057 Florida propagations in 2019–20 (Rosson, 2020).

At IVIA (Valencia, Spain), conventional sexual hybridization to develop superior new citrus rootstocks has been underway since the 1970s (Martínez-Cuenca et al., 2016). During the later stages, the IVIA program emphasized and documented information on screening and selection of hybrids for resistance to salinity (Forner-Giner et al., 2009), iron deficiency (Llosa et al., 2009), and water stress (Rodriguez-Gamir et al., 2010). Validating tolerance to citrus nematode *Tylenchulus semipenetrans* (Verdejo-Lucas et al., 1997) and *Phytophthora* species found in Spain has also been an important part of rootstock selection, along with highly uniform seedling propagation (Forner et al., 2003). The rootstocks released from the program include F-A 5, F-A 13, F-A 418, and F-A 517, with the latter two being considered dwarfing (Forner-Giner et al., 2014). Of these, F-A 5 has had the most commercial interest, with considerable plantings using this rootstock in Spain and nearby citrus production regions.

In the 1960s, the CRA-Research Center for Citriculture and Mediterranean Crops (CRA-ACM) initiated a conventional citrus rootstock breeding program in Sicily, Italy. Selections were made from among progeny from crosses for tolerance to "mal secco" and *Phytophthora*, and for nucellar embryony and vigor, followed by field trials (Russo and Reforgiato Recupero, 1984; Reforgiato Recupero and Russo, 1992). Out of this work, CREA (Acireale, Italy) released three hybrids of *C. latipes* (Tan.) × *P. trifoliata* (F5P12, F6P12, F6P13) that demonstrated high yield in field trials with several different scions (Reforgiato Recupero et al., 2009). In a recent rootstock trial with triploid Mandared scion in Siracusa (Italy), this team found that some of the rootstocks from California (including C35) were superior to the CREA hybrids (Caruso et al., 2020).

Citrus production in Brazil is primarily on Rangpur rootstock, which is well suited to rainfed cultivation and the primary crop of that region, juice oranges. But there is still great interest in diversifying rootstock use and finding better rootstocks for specialty scions, different production areas in the region, and to cope with HLB and other specific problems, motivating active breeding and evaluation under EMBRAPA and at The Sylvio Moreira Citriculture Center (Institute Agronomic of Campinas, Cordeirópolis), as well as work under Fundecitrus, IDR-Paraná (Rural Development Institute of Paraná), and University of São Paulo (ESALQ – USP). Hybrid and clonal rootstocks from Brazil and other countries are evaluated for nursery performance (Marques et al., 2021), drought (Santana-Vieira et al., 2016), and tree size control (Costa et al., 2021). Numerous field trials have been used to compare performance of the many rootstocks, with superior rootstocks identified for the different production situations (Cantuarias-Avilés et al., 2010; Girardi et al., 2017; Carvalho et al., 2019; Costa et al., 2020b; de Carvalho et al., 2021). Focused studies were conducted to map quantitative trait loci (QTL) in citrandarins associated with host response to infection by CLas (Soratto et al., 2020) and *Phytophthora parasitica* (Lima et al., 2018), and to identify mechanisms of tolerance to HLB (Curtolo et al., 2020). A series of non-parametric indices were evaluated for selection of superior hybrid rootstocks, and a ranking index was identified as the most useful for selecting productive and drought-tolerant rootstocks (Costa et al., 2020a).

To date, the only hybrid citrus rootstocks from breeding programs that have gained major commercial importance (used for more than 5% of nursery production over several years in at least one important crop region; as reported in Bowman and Joubert, 2020) besides Carrizo and Swingle (**Table 1**), are Benton, C35, and Kuharske citranges (*C. sinensis* × *P. trifoliata*), X639, F-A 5, US-812, US-897, and US-942 citrandarins (*C. reticulata* × *P. trifoliata*), US-802 (*C. maxima* × *P. trifoliata*), and MxT [(*C. paradisi* × *C. reticulata)* × *P. trifoliata*)]. The other rootstocks of major commercial importance are naturally occurring species or selections, including Cleopatra and Sunki mandarins, clones of trifoliate orange (*Poncirus trifoliata)*, Rangpur, Rough lemon, Volkamer, and Ziyang Xiangcheng, and these all exhibit nucellar polyembryony. All the commercially important products from breeding programs are F1 hybrids of trifoliate orange with citrus, and all possess nucellar polyembryony, a trait that is obtained in a large portion of progeny from crosses of trifoliate orange with many citrus species. Efforts have been made to expand the range of parentage included in new rootstock hybrids, such as the *C. latipes* × *P. trifoliata* hybrid rootstocks from CREA-Italy (Reforgiato Recupero et al., 2009), but none of these have yet gained commercial acceptance.

Over the last 35 years, research efforts to develop improved citrus rootstocks have expanded at many institutions to include new methodologies (Salonia et al., 2020), including ploidy manipulations, somatic hybridization, genetic transformation, and genome editing. Selections of rootstock clones with a doubling of chromosomes, or autotetraploids, can often be visually identified among groups of seedlings and have received interest for more than 80 years (Lapin, 1937; Russo and Torrisi, 1951; Barrett and Hutchison, 1978; Bowman et al., 1991; Ollitrault et al., 2020). There is evidence that some autotetraploids have greater stress tolerance than their diploid twin (Allario et al., 2013; Oliveira et al., 2017; Oustric et al., 2017), but none of these tetraploid selections have become important rootstocks in commercial use. Considerable effort has been invested and

[1]https://programs.ifas.ufl.edu/plant-breeding/citrus/

TABLE 1 | Major citrus rootstocks of the world, with regions of primary use, parentage (for those from known crosses), date commercial use began, and uniformity of seed propagation.

Rootstock	Current regions of primary commercial use[a]	Parentage	Beginning of use as rootstock	Uniform seed propagation
Benton	SU	*Citrus sinensis* × *Poncirus trifoliata*	1990	Yes
Carrizo	NCSEFMAU	*C. sinensis* × *P. trifoliata*	1934	Yes
Cleopatra	NCSEMU	*C. reticulata*	Before 1900	Yes
C35	NCSEFMU	*C. sinensis* × *P. trifoliata*	1986	Yes
F-A 5	E	*C. reticulata* × *P. trifoliata*	2003	Yes
Kuharske	N	*C. sinensis* × *P. trifoliata*	Before 2000	Yes
Macrophylla	NEMU	*C. macrophylla*	Before 1960	Yes
MXT	F	(*C. paradisi* × *C. reticulata*) × *P. trifoliata*	1992	Yes
Trifoliate orange	NCSEMAU	*P. trifoliata*	Before 1900	Yes
Rangpur	CSA	*C. limonia*	Before 1900	Yes
Rough lemon	NCFA	*C. jambhiri*	Before 1900	Yes
Sour orange	NCSEMA	*C. aurantium*	Before 1850	Yes
Sunki	S	*C. sunki*	Before 1900	Yes
Swingle	NCSEFAU	*C. paradisi* × *P. trifoliata*	1974	Yes
US-802	N	*C. maxima* × *P. trifoliata*	2007	Yes
US-812	NM	*C. reticulata* × *P. trifoliata*	2001	Yes
US-897	N	*C. reticulata* × *P. trifoliata*	2007	Yes
US-942	N	*C. reticulata* × *P. trifoliata*	2010	Yes
Volkamer	NCSEFMAU	*C. volkameriana*	Before 1970	Yes
X639	NCF	*C. reticulata* × *P. trifoliata*	2000	Yes
Ziyang Xiangcheng	A	*C. junos*	2010	Unknown

[a]*N, North America; C, Central America and Caribbean; S, South America; E, Europe; F, Southern Africa; M, North Africa and Middle East; A, Asia; U, Australia.*

progress made in somatic hybridization directed at rootstock improvement at University of Florida (Grosser et al., 1998), and in France (Dambier et al., 2011) and Spain (Ruiz et al., 2018), after the technique was first demonstrated in Japan (Ohgawara et al., 1985). Most initial work with somatic hybrids was to create allotetraploid hybrids among existing rootstocks (Dambier et al., 2011) and with other species (Grosser et al., 1996). Subsequent work included the creation of cybrids (Moreira et al., 2000) and has followed with sexual hybridization among the tetraploids (Grosser et al., 2015). New rootstocks that are the product of somatic hybridization have been released in Florida (Kunwar et al., 2021), although so far none of those from Florida or other programs have become of major commercial importance.

Agrobacterium-mediated genetic transformation has been applied toward improvement of citrus rootstocks for more than 25 years (Cervera et al., 1998; Benyon et al., 2013), but significant challenges remain to the development and commercial acceptance of the GMO products (Song et al., 2019). Current public attitudes toward the products of *Agrobacterium*-mediated genetic modification seem to limit applications in fruit crops (Lucht, 2015); but the potential to use GMO rootstocks to improve the health and productivity of trees with non-GMO scions has been proposed as one way to avoid this problem (Haroldsen et al., 2012; Song et al., 2015). Methods to excise the selectable markers in transgenic citrus plants have been demonstrated (Zou et al., 2013) and proposed as another way to alleviate GMO restrictions and reduce public concerns. Introduction of early flowering genes by genetic transformation to accelerate the breeding cycle of fruit tree crops, followed by backcrossing to remove those genes before commercialization of the new cultivars, have also been proposed as a method to use *Agrobacterium*-mediated transformation for genetic improvement and circumvent GMO-related concerns (Petri et al., 2018). Induction of early flowering through transient expression by a viral vector has been demonstrated in citrus (Velazquez et al., 2016), and is yet another approach to using an early-flowering transgene to accelerate citrus breeding, while potentially avoiding a GMO-cultivar product.

Genome editing avoids some of the regulatory limitations of genetic transformation and has been demonstrated in citrus (Jia and Wang, 2014). Some progress has also been made in efforts to use genome editing to improve disease resistance (Peng et al., 2017; Jia et al., 2019; Wang et al., 2019). Although numerous groups have made very large investments of resources into *Agrobacterium*-mediated transformation and genome editing of citrus (Poles et al., 2020), as of now, no citrus rootstocks derived from GMO technology or genome editing have become commercially available. While new technologies offer abundant options for continuing work, none has yet proven successful in creating rootstocks of commercial importance, and it seems likely that conventional breeding will continue to have the primary role in the development of the next generation of citrus rootstocks in the coming decade.

NEED FOR BETTER ROOTSTOCKS AND A NEW STRATEGY

Citrus rootstock breeding programs are expensive and require very long-term investment that is generally not suited to

the objectives of private industry, leaving such research to government or university sponsored programs. Despite considerable effort toward developing and testing new citrus rootstocks at numerous institutions and citrus growing regions over the past 100 years, inadequate rootstocks are still used for a large portion of world citrus production. Rootstock trials in different regions demonstrate, repeatedly, that trees on some particular rootstocks show better tolerance to disease and stress, and yield more and better quality fruit than other rootstocks (Louzada et al., 2008; Rodriguez-Gamir et al., 2010; Bowman et al., 2016a; Cruz et al., 2019; Kunwar et al., 2021; Singerman et al., 2021). Within a regional rootstock trial, often the best performing rootstocks are not those that are in most common commercial use. One impediment to adoption of better rootstocks in commercial plantings is the long life of a citrus tree and the expectation of multiple-decade production from a tree. It is often considered uneconomical to replace an existing mature bearing tree, even if the replacement tree might eventually become more productive. However, of greatest importance in limiting the development and commercial adoption of superior new rootstocks are: (1) the limited genetic diversity of germplasm used in creating new hybrids and compared in individual field trials, (2) the limited number of rootstocks in trials and locations of trial comparisons among rootstocks, so that growers do not have enough information to be sure about the best choice in broader regions, (3) the slow pace of new rootstocks being available from commercial nurseries, when obligate apomictic seed propagation is required, and (4) the inability to select the best genetic combinations at an early stage of rootstock development, resulting in large resource utilization in testing many unfavorable candidate rootstocks.

To address these problems with new rootstock development and the adoption of superior rootstocks by industry, the USDA citrus breeding program in Fort Pierce, FL (United States) began a new strategy for rootstock development in 2006. The new strategy was termed "SuperSour" because the target was to create a superior new hybrid rootstock that possesses the best rootstock traits of sour orange (*C. aurantium*) along with superior tolerance to HLB and other biotic and abiotic problems and superior production of high-quality fruit, while avoiding sensitivity to CTV-related tree decline. The strategy includes *C. maxima* and *C. reticulata* (the two parental species of sour orange) in most hybrids, along with *P. trifoliata* and other species to introduce additional positive rootstock traits. The primary features of this new SuperSour strategy are: (1) the expanded gene pool used in new rootstock hybrids, including germplasm that was not previously used (such as *C. maxima*) because it does not transmit nucellar polyembryony to a high portion of progeny, (2) accelerated field testing of new hybrids by propagating the hybrids with stem cuttings, rather than waiting for fruiting and seeds, (3) expanded concurrent stage 1 field testing of new hybrids in multiple production sites and multiple planting dates, that can be cross-compared by using the same standard rootstocks in each trial, (4) release of new rootstocks based on superior performance in multiple field trials, and without a requirement for propagation by nucellar polyembryony, and (5) assembly of standardized multi-year performance data on hundreds of hybrid rootstocks from multiple trials, which can be used to genetically map important rootstock traits and develop molecular markers to streamline future rootstock breeding.

The new strategy includes evaluation and testing for nursery traits, and tolerance or resistance to abiotic and biotic factors. But this testing is done concurrently with field trials of the candidate hybrid rootstocks, rather than as a prerequisite for inclusion in field trials. While nursery traits like nucellar polyembryony and vigorous nursery growth are valuable, they do not directly affect the field performance of the rootstock. Tolerance or resistance to abiotic and biotic factors are valuable for field performance, but survival, health, and fruit productivity in the field is the combination of numerous interrelated traits which are best evaluated in the true complex field environment. Our strategy is based on the observation that the best rootstock in the field is that which combines optimum levels of abiotic tolerance to numerous factors, biotic resistance to numerous diseases, moderate growth traits, and favorable physiological influence on tree nutrition and fruit. This optimum combination is most efficiently identified by direct field trials involving all factors relevant to the production environment, rather than focused testing of individual traits. Concurrent or subsequent testing on those individual factors is most efficient when it can be focused on those few individual new hybrids that clearly exhibit the most outstanding field performance. Information from the focused testing can then be used to define the strengths, weaknesses, and limits of likely production conditions for each of those candidate rootstocks.

EXPANDING THE GENE POOL BY ELIMINATING NUCELLAR EMBRYONY AS A REQUIRED TRAIT

During the earliest citrus cultivation, trees were primarily grown as seedlings, and nucellar polyembryony was a prerequisite in selection of which citrus types were repeatedly used over time. During the 19th century, the advantages of grafting for maintaining fruit traits, promoting early flowering, and allowing the combination of the best fruit traits with the best root traits became recognized. Using a rootstock (such as sour orange) that propagates easily and uniformly from seed was a big advantage in obtaining a large planting of easily managed productive trees. Consequently, early citrus nursery production quickly focused on rootstock varieties that produced genetically uniform seedlings by nucellar polyembryony. By the time attention was being given to creating new hybrids that might be used as rootstocks, the great convenience of a citrus rootstock that could be propagated uniformly by seed was demanded by citrus nurseries and has heavily focused rootstock breeding efforts for most of the past 100 years. Crosses in which at least one parent did not have and transmit nucellar polyembryony to progeny were avoided for rootstocks, and progeny were not evaluated as potential rootstocks until they had fruited and seedling reproduction could be assessed. Moreover, hybrids that did not themselves exhibit nucellar polyembryony in their seeds were excluded from further testing. Although there are a few examples of citrus used as rootstocks which had less than 50% nucellar seed (Bowman et al., 1995; Rodriguez et al., 2005) and vegetative propagation was possible for most germplasm (Bowman et al., 1997), clones

that did not have a high incidence of nucellar seed production were generally not tested as potential rootstocks, and none have become of major commercial importance.

Beginning from previously described methods for stem cutting propagation of citrus (Ferguson et al., 1985), modified methods were developed in the USDA citrus rootstock program to expand both the genetic diversity of hybrid clones that could be used and to increase the percentage of success for the stem cuttings. Initial work resulted in vegetative propagation of numerous new hybrids for field trials, and eventually resulted in the commercial release of three new HLB-tolerant hybrid rootstocks that do not exhibit nucellar polyembryony and cannot be uniformly propagated by seed (Bowman and McCollum, 2015; Barcelos-Bisi et al., 2020). The methodology for citrus stem cuttings evolved through this process and has been described in detail (Bowman and Albrecht, 2017). The new methods are broadly applicable to all citrus types, although frequency of rooting varies by clone. We have found that more than 95% of all clones and hybrids can be successfully propagated by stem cuttings using these methods, allowing it to be employed for replicated field trial testing of nearly any citrus clone as a rootstock. In a recent propagation cycle, more than 5000 single node stem cuttings were made from a diverse collection of 72 citrus cultivars and new hybrids. Three months later, 71 of the 72 clones had produced some growing rooted cuttings, and growing cuttings were obtained from 58% of the cuttings overall. Only 3% of the clones yielded less than 10% healthy plants. Although a high rate of success in stem cutting propagation would be needed for commercial use, even 10% success in rooting is adequate to effectively propagate new rootstock hybrids for field trials. For reference, Swingle, Cleopatra, and sour orange yielded 99%, 56%, and 33% of healthy plants from cuttings, respectively.

The stem cutting methodology is most suitable for small-scale propagation of a rootstock clone (10–1000 individual rootstock plants), while tissue culture propagation (Carimi and De Pasquale, 2003; Prakash and Sharma, 2018) is more suitable for larger-scale (>5,000 plants) production of a rootstock. Historically, there was concern about whether the characteristics and health of rootstocks propagated by stem cuttings and tissue culture would be as good as that of rootstocks propagated by seed. But rootstocks propagated by all three methods (seed, cuttings, and tissue culture) have been studied intensively, with clear indication that nursery and field performance are primarily determined by the genetic traits of the rootstock cultivar, and only minimally affected by propagation method (Albrecht et al., 2017, 2020; Pokhrel et al., 2021). In one current field trial with Valencia scion and four rootstock clones, a comparison of the rootstocks propagated by nucellar seedlings, stem cuttings, and tissue culture (**Figure 1**) demonstrated a significant effect of rootstock clone on young tree growth and canopy health, but no significant effect from rootstock propagation type (**Table 2**). During the 12-month period from July 2019 to June 2020, Florida Department of Agriculture and Consumer Services (FDACS) records indicated that 20% of new propagations (770,000 trees) in Florida used tissue culture propagated rootstock plants, and 6% of new propagations (220,000 trees) used rootstocks propagated by stem cuttings (Rosson, 2020). Although seed propagation is still dominant in commercial use and less expensive than propagating by cuttings or tissue culture, there is a growing broad acceptance of citrus rootstocks propagated by alternative vegetative methods.

FIGURE 1 | Valencia sweet orange nursery trees at planting time on US-942 rootstock propagated by seed (top), tissue culture (center), and stem cuttings (bottom). Rootstock propagation type appears to have little effect on citrus rootstock performance.

TABLE 2 | Canopy volume, increase in trunk cross sectional area (ΔTCSA), and canopy health rating of 'Valencia' trees on different rootstocks propagated by seed (SD), cuttings (CT), and tissue culture (TC) after 18 months in the field.

Factor	Canopy volume (m^3)	ΔTCSA (mm^2)	Canopy health rating
Propagation method			
SD	0.248	518	4.25
CT	0.236	496	4.23
TC	0.218	444	4.15
P-value	0.3022	0.0904	0.8282
Rootstock cultivar			
US-1516	0.275 a	517 a	4.45 a
US-812	0.244 a	512 a	4.20 a
US-897	0.131 b	318 b	3.67 b
US-942	0.287 a	599 a	4.53 a
P-value	<0.0001	<0.0001	<0.0002
Propagation method × rootstock			
P-value	0.3084	0.1029	0.8668

Different letters within columns indicate significant differences according to Tukey's HSD test at 5% level.

Some species of citrus (especially those species of hybrid origin, such as *C. sinensis*, *C. aurantium*, and *C. paradisi*) produce predominantly polyembryonic seeds. Other species and selections lack nucellar embryony and produce entirely zygotic seeds, including *C. maxima* (pummelo), *C. medica* L.(citron), some *Citrus* species in the subgroup Papeda, some mandarin species (including *C. clementina* Hort. Ex Tan., *C. tachibana* Tan., some *C. reticulata*), and species in the sexually compatible genus *Microcitrus* (Swingle and Reece, 1967). Evidence has been presented that suggests the simple genetic control of polyembryony in some crosses (Cameron and Soost, 1979), while in other crosses the genetic basis of the trait appears to be

more complex (Garcia et al., 1999; Kepiro and Roose, 2010). In breeding practice, hybrid progenies that include even one monoembryonic (zygotic) parent often have few hybrids that possess a level of nucellar polyembryony and apomictic seed that would be acceptable for commercial uniform seed propagation. Although methods are available that allow separation of zygotic from apomictic seedlings in the nursery (Barcelos-Bisi et al., 2020), these are not economically practical at a commercial nursery scale for many types of hybrids. In addition, some hybrids produce few fruit or few seeds per fruit, another way in which outstanding rootstocks may not be suitable for seed propagation.

As described above, in previous citrus rootstock breeding efforts, one of the factors considered essential for a citrus rootstock was the ability to be propagated uniformly by seed (Bitters, 1986), and germplasm that did not transmit nucellar polyembryony to hybrids was rarely used as a parent in rootstock breeding. Under the new SuperSour rootstock breeding strategy, hybridization for new rootstocks is expanded to include parental material that does not exhibit nucellar polyembryony, or does not transmit this trait to progeny. During the early stages of crosses under the new strategy, the gene pool was expanded primarily by including *C. maxima*, *C. ichangensis*, *C. latipes*, mono-embryonic mandarins, and *Microcitrus* spp. among the parental material. Based on our work with vegetative propagation of rootstocks by stem cuttings and tissue culture, any rootstocks with superior field performance can be effectively used on a commercial scale without any need for seed propagation in the commercial nursery.

ACCELERATED FIELD TESTING BY PROPAGATING JUVENILE HYBRIDS WITH STEM CUTTINGS

In addition to the limitation on genetic diversity that resulted from a focus on the need for nucellar seed, this requirement also resulted in a large delay in the initiation of field-testing of new hybrids as rootstocks. In previous rootstock breeding efforts, new hybrid progeny from crosses were planted into the field for "fruiting out" as a first stage in the selection process. It was only after those hybrids produced fruit, that new selections were propagated as seedlings, and entered into specialized testing and replicated field trials (Hutchison, 1976, 1985; Cameron and Soost, 1986). This delay in testing of new rootstock hybrids until seed were produced (and confirmed to be genetically uniform), varied by parentage and conditions, but typically was at least 5 years, and often 10–15 years.

With the SuperSour strategy, young hybrid seedlings are selected during the first 1–2 years in the greenhouse for strong healthy growth, and propagated by stem cuttings for further testing (Bowman and Albrecht, 2017). This eliminates the 5- to 15-year delay to begin testing and replicated field trials with new hybrid rootstocks. Under the new strategy, replicated trees of each rootstock are produced on healthy rooted cuttings, using standard budding methods and a standard sweet orange scion (Valencia 1-14-19 or Hamlin 1-4-1) in a certified greenhouse nursery. The sweet orange trees are subsequently planted into replicated field trials alongside trees on other new hybrid rootstocks and commercial standard rootstocks in one of the three main Florida production regions (East Coast, Central Ridge, Southwest). Propagations of the new SuperSour hybrid clones are also used concurrently to evaluate tolerance to specific diseases and abiotic factors in the field and greenhouse (Bowman and Garnsey, 2001; Albrecht and Bowman, 2004; Graham et al., 2007; Bowman and Albrecht, 2020), and to maintain a clean greenhouse source of vegetative material for continuing propagation and study. Fruiting trees of rootstock selections that demonstrate outstanding field performance may be used as parents in the next generation of rootstock hybrids within the USDA breeding program.

Clean greenhouse source trees of hybrid rootstock selections that demonstrate outstanding field performance are used to submit the clone to the FDACS Citrus Budwood Program[2] to establish certified clean source material for eventual Florida industry use. Certified clean source material of new hybrid rootstocks that appear to have potential in other production regions are submitted to the California Citrus Clonal Protection Program[3] and the USDA National Clonal Repository for Citrus, for inclusion in those programs. Propagations of the new SuperSour hybrid clones are also used to plant trees into the field for fruiting out and evaluation of nucellar polyembryony, and the potential for commercial seed propagation. Although the potential for uniform seed propagation is eventually determined for the SuperSour hybrids, it is not a selective factor in determining which hybrids will go into replicated field trials, or a key factor in decisions about which selections may be released for commercial use.

The use of stem cuttings to conduct replicated evaluations of new hybrid rootstocks and establish replicated field trials has already resulted in the successful identification of five new promising rootstocks that were released for commercial use in Florida (Bowman and McCollum, 2015). Subsequent evaluation of seeds from fruiting trees of the five rootstocks revealed that two of them can be uniformly seed propagated, while the other three cannot (Barcelos-Bisi et al., 2020). However, because of the ease of alternative propagation by stem cuttings or tissue culture, uniform seed propagation is not an important factor in evaluating the commercial potential for these five new hybrid rootstocks.

OTHER ADVANTAGES OF ELIMINATING FOCUS ON NUCELLAR SEED PROPAGATION

In addition to allowing the expansion of the gene pool for rootstock breeding and the rapid initiation of field trials, eliminating a focus on nucellar seed propagation presents at least two other advantages for the SuperSour strategy. One advantage is that it allows greater focus on other traits of horticultural importance within the rootstock selection process. The previous focus on nucellar polyembryony as a primary

[2]https://www.fdacs.gov/

[3]https://ccpp.ucr.edu/

selective factor resulted in all other factors (including resistance to biotic and abiotic disease, and fruit productivity) being of secondary importance during the selection process. New hybrids were only considered as potential rootstocks if they possessed the trait of nucellar polyembryony, and consequently many hybrids with potentially very good disease resistance and very high productivity were never considered for further study. By eliminating nucellar embryony as a selective factor, more focus can be given to selection for traits with much greater horticultural importance.

The second advantage is that the recognition of vegetative propagation (cuttings or tissue culture) as an acceptable alternative for citrus rootstocks provides opportunities for much more rapid scale-up of nursery production with the newest outstanding rootstocks. When using seed propagation, there is a delay of several years after seed source trees for a superior rootstock are established and significant quantities of seed are available. We might anticipate that newly planted seed trees for a rootstock will produce no fruit in the first season, 10 fruit per tree after 24 months, and 30 fruit per tree after 36 months. If 250 seed trees were initially planted (about half a hectare) and the rootstock produces 10 seed per fruit, that would equal about 25,000 rootstock seedlings that could be grown after 24 months, and another 75,000 seedlings after 36 months. Estimates of the rate of micropropagation can vary widely, but starting with those same 250 buds to create the seed trees, and using an estimate of citrus tissue culture shoot multiplication at 3.8 × per 4 weeks (Sen and Dhawan, 2010), it would be possible to create more than one million rootstock plants in less than 12 months. Vegetative propagation by tissue culture of new citrus rootstocks allows a much more rapid scale-up of plant production in the nursery than is possible by nucellar seed production.

Taken together, while apomictic seeds of citrus rootstocks are convenient for commercial nursery production, the focus on this trait in commercial rootstock selection has eliminated large segments of the citrus gene pool from use in development of new hybrid rootstocks, greatly delayed the beginning of testing new rootstocks, and diverted focus away from the more important horticultural traits. Eliminating this focus as a selection factor under the SuperSour strategy is a major positive change that will improve the opportunity to identify new rootstocks with superior horticultural traits and significantly accelerate the large-scale commercial use of the newest and best rootstocks.

EXPANDED CONCURRENT STAGE 1 FIELD TESTING OF NEW HYBRIDS

Most documented systematic comparisons of citrus rootstocks in field trials were established as individual trials, and compared an assortment of different existing and new rootstocks in a replicated planting over at least four harvest seasons (Wutscher and Bowman, 1999; Forner-Giner et al., 2003; Louzada et al., 2008; Cantuarias-Avilés et al., 2010; Bowman et al., 2016b; Continella et al., 2018). Typically, trials have contained 4–7 statistical replications of 10–24 different rootstocks, often including existing rootstocks common to the area and selected new or imported rootstocks from other regions. In many situations this was useful and helped define the best among current rootstock options. However, these individual trials with relatively few rootstocks are not suitable for a strategy to compare hundreds of new rootstocks with a limited amount of time and resources. Other trials focused on comparisons of larger numbers of rootstocks were much larger in total area and required much larger commitments of resources to maintain the trials and collect the needed data (Castle et al., 2011; Kunwar et al., 2021).

The new SuperSour strategy employs a series of linked trials to greatly increase the number of rootstocks that can be compared among each other. The trials are linked by having a common scion (sweet orange), a similar experimental design, and several common rootstocks as points of reference, to allow relative comparisons among the rootstocks in all the trials. This provides the opportunity for the effective concurrent testing of hundreds of rootstocks in multiple trials, and generates performance data that can be systematically compared between the trials. Between 2014 and 2021, there were 15 of these linked SuperSour trials with sweet orange scion planted in Florida, containing 350 new hybrid rootstocks along with four standard rootstocks for cross-comparison in all the trials. Reference of results in each trial to the standard rootstocks will allow multi-trial comparisons of rootstocks and improve selection of new rootstocks that are best overall, not just in individual trials. The average number of new hybrid rootstocks in each trial is 50, average rootstock replications per trial is nine, and most of the 350 hybrid rootstocks are included in more than one trial.

Two of the 15 trials have been chosen to illustrate the strategy and results obtained from the trials. The two representative trials are in adjacent sections at the USDA Picos Farm in Fort Pierce, Florida, which is on the East Coast of Florida where poorly drained sandy soils are typical (Mylavarapu et al., 2019). One of the trials was planted in October 2014 and the other in October 2015, and named 'Picos 2014' and 'Picos 2015,' respectively (**Figure 2**). Each trial was planted on 12 rows, with about 640 trees per trial. The trials are planted on leveled double row beds, with good drainage and microsprinkler irrigation, as is standard practice for this area. Rootstocks for the trials were propagated by stem cuttings (Bowman and Albrecht, 2017) from the hybrid seedlings, and trees produced by budding with certified Valencia 1-14-19 sweet orange budwood using standard methods in the certified greenhouse citrus nursery at the US Horticultural Research Laboratory in Ft. Pierce, FL, United States. Management of trees in the two blocks employed common fertilization, weed, and pest control practices for citrus in this area. As is normal for field plantings in Florida citrus production areas where CLas is endemic (Graham et al., 2020), PCR surveys of trees in the block indicated that within the first 3–4 years after planting, 100% of trees in the trials were infected with CLas. A mild strain of CTV is also endemic in the area of these trials, although usually there are no negative effects on sweet orange trees from infection.

Detailed annual performance information on each replication is collected from the SuperSour field trials, including measurements of tree survival and size, canopy health, quantity of fruit produced, and fruit quality (brix, acid, percent juice, color, and fruit size). Performance traits of the greatest interest

FIGURE 2 | Aerial image in May 2021 of the two rootstock trials with Valencia sweet orange scion, planted with 1287 trees (Photo by R. Niedz).

in citrus rootstock trials, and approach to analysis of results can vary by situation and focus. However, generally the traits tree health, fruit yield, yield efficiency, pounds solids per box (PSB), juice color, and brix:acid (BA; soluble solids:titratable acidity) ratio are of great importance in assessing the performance of rootstocks in Florida field trials with sweet orange scion being grown for juice production. Within both trials, significant differences were observed among the rootstocks for all six of these traits (see **Supplementary Tables 1, 2**).

To illustrate overall tree performance in the two trials, results were transformed to make relative comparisons with the standard rootstock sour orange within each trial for the traits: (1) cumulative fruit yield (over seasons 4–6), (2) canopy health (subjective score of canopy density and leaf color), and (3) yield efficiency (ratio of fruit yield:canopy size). Results are presented in a bubble chart (**Figure 3**), with all values transformed to make sour orange values zero for cumulative yield and canopy health. Values for yield efficiency were transformed so that sour orange yield efficiency produced a medium-size bubble). In the chart, the mean value for each rootstock relative to sour orange was represented by a single bubble, and results are combined from the two trials. Rootstocks which induced relatively higher or lower values for cumulative yield, canopy health, and yield efficiency than sour orange are represented according to those differences by position on the x-y axis and by bubble size. In total, **Figure 3** compares values for sour orange rootstock with three other standard rootstocks (Swingle citrumelo, Cleopatra [*C. reticulata*], and Ridge [*C. sinensis*]) and 97 SuperSour hybrid rootstocks. Included in this summary are data from 59 hybrids plus the four standard rootstocks in Picos 2014, and 44 hybrids plus the four standard rootstocks in Picos 2015 (six of the hybrids were in both trials). In these two trials, three different types of parental combinations were included among the new SuperSour hybrids being compared, and are indicated by bubble color on the chart:

- *C. maxima* × *C. reticulata*, or the reverse
- *C. maxima* × (*C. reticulata* × *P. trifoliata*)
- *C. reticulata* × (*C. maxima* × *P. trifoliata*).

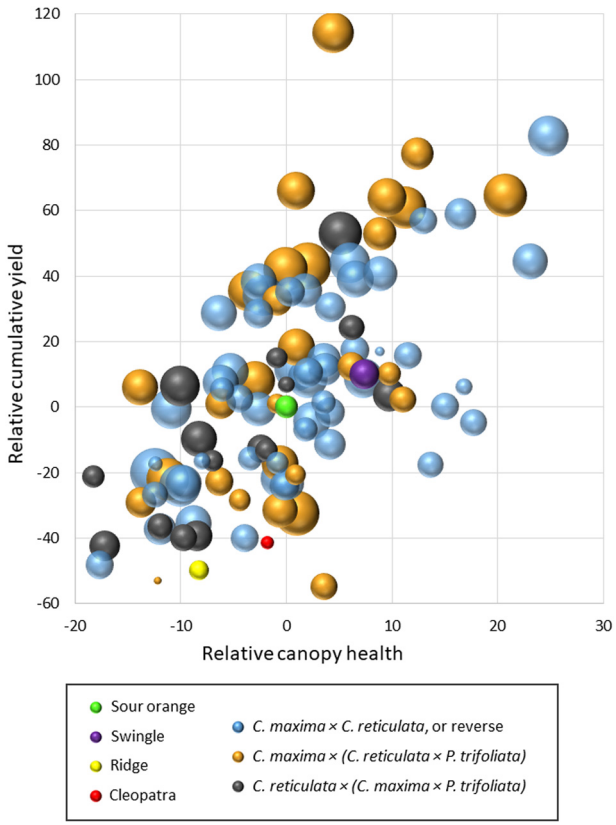

FIGURE 3 | Comparison of cumulative yield, canopy health, and yield efficiency (bubble size) for 97 new SuperSour hybrid rootstocks relative to standard sour orange rootstock and three other commercial rootstocks in two trials with Valencia scion, planted in 2014 and 2015.

More than half of the progeny from all three parental combinations were superior to sour orange rootstock for cumulative yield, canopy health, and yield efficiency. Among the other three standard rootstocks in the trials, Swingle was slightly better than sour orange for the three traits, while the other two (Cleopatra and Ridge) were worse. This matches well with commercial use, as Swingle and sour orange are among the five most used rootstocks in Florida, while Cleopatra and Ridge have relatively little commercial use. Some of the new SuperSour hybrids demonstrated very large advantages in cumulative yield and yield efficiency as compared with the standard rootstocks. This suggests good potential to identify superior new commercial rootstocks among these progeny types. The comparison of performance from progenies of the three parental combination types may suggest relative differences in potential from different types of crosses, which will be the subject of further study.

A similar comparison was made in these same two trials on the influence of rootstock on fruit quality of the Valencia sweet orange scion during the 2019–21 seasons. Results within each trial were transformed to make relative comparisons with the standard rootstock sour orange for the traits: (1) PSB, (2) juice color (CN color scale), and (3) juice BA ratio. Results are presented in a bubble chart (**Figure 4**), with all values

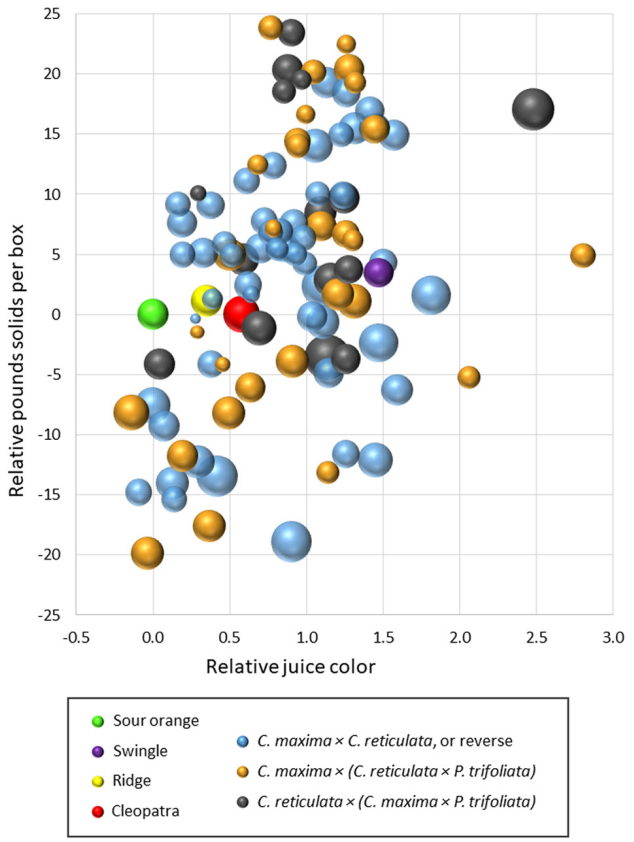

FIGURE 4 | Comparison of fruit pounds solids per box, juice color, and brix/acid ratio (bubble size) for 97 new SuperSour hybrid rootstocks relative to standard sour orange rootstock and three other commercial rootstocks in two trials with Valencia scion, planted in 2014 and 2015.

transformed to make sour orange values zero for PSB and juice color. Values for BA ratio were transformed so that sour orange BA ratio produced a medium-size bubble. As in **Figure 3**, the mean value for each rootstock relative to sour orange was represented by a single bubble, and results are combined from the two trials. **Figure 4** compares fruit quality values over three harvest seasons for sour orange rootstock with three other standard rootstocks (Swingle, Cleopatra, and Ridge) and 97 SuperSour hybrid rootstocks. More than half of the SuperSour hybrids induced a higher PSB than sour orange, and the PSB value for the other standard rootstocks were similar to that for sour orange. For influence on Valencia juice color, sour orange was among the worst of the rootstocks, and nearly all of the SuperSour hybrids (and the other three standard rootstocks) exhibited a superior influence on juice color. Most notably, Swingle was among the rootstocks that had the strongest positive influence on juice color. Although some of the hybrids were similar, sour orange was among the best of the rootstocks for influence on BA ratio, suggesting that this trait may be the greatest challenge to achieve for a superior new rootstock.

It appears from these results that many of the hybrid rootstocks among these three different types of SuperSour parental combinations may be superior in many respects to the common commercially used rootstocks in Florida, like sour orange and Swingle. Relative comparisons among these two trials suggest that results from the larger group of 15 trials can be cross-compared and used effectively to identify those rootstocks with superior positive attributes for potential commercial release. Comparisons of rootstock performance under different conditions at different locations is included as a part of the SuperSour strategy, but is more complex, and is not included in this illustration. Individual relative deficiencies of particular hybrids that are otherwise outstanding can be clearly identified and evaluated for relative significance in commercial use. In some cases, relative weaknesses (for example, relatively low color score) might be judged of minor significance for a rootstock that otherwise induced high yield, high yield efficiency, and high PSB.

IDENTIFY SUPERIOR PARENTAL COMBINATIONS AND MAP ROOTSTOCK TRAITS

One large long-term benefit of the new SuperSour strategy, and the concurrent testing of hundreds of related hybrid rootstocks from several different parental combinations, is the opportunity to identify those parental combinations that have the highest likelihood to produce progeny with particularly important rootstock traits or combinations of important rootstock traits. Although previous field trials and resultant datasets related to citrus rootstock traits were inadequate to evaluate the combining ability of potential citrus parents (the ability to transmit positive rootstock attributes to progeny), accurate estimates of general and specific combining ability are widely considered a critical tool in plant breeding (Allard, 1960; Oakey et al., 2006; Fasahat et al., 2016). Information about the combining ability of numerous specific citrus parents or types of parents would be an immensely valuable tool to help guide the next generations of citrus rootstock breeding. Among the 97 hybrid progeny in the two-trial illustration (Picos 2014 and Picos 2015), there were large differences in mean values for the three progeny groups that were compared. For example, the population of *C. maxima* × (*C. reticulata* × *P. trifoliata*) hybrid progeny tested in those two trials had a mean value for cumulative yield of plus 13.8 units, while the mean cumulative yield value from the progeny of the cross *C. reticulata* × (*C. maxima* × *P. trifoliata*) had a mean value of minus 8.1 units. While this is only a preliminary comparison from a partial dataset, it suggests that one parental combination is likely to create hybrids with higher yield than the other parental combination. The progeny groups developed under the SuperSour strategy, with corresponding field trials, will generate the needed datasets to identify optimum parental combinations and utilize estimates of combining ability as a tool to help guide next-generation citrus rootstock breeding.

Related to knowledge about combining ability, the extensive datasets for rootstock performance information of a large group of citrus hybrids can also be used to effectively map genes associated with important rootstock traits on the citrus genome.

Molecular markers, association mapping, and quantitative trait locus mapping are common tools in plant breeding of annual crops, made practical by the use of test crosses and very short generation times (Xu and Crouch, 2008; Ibrahim et al., 2020). Recent advances in data analysis demonstrate that similar mapping of genes can be accomplished from unbalanced and diverse multi-environment field trial data (Rife et al., 2018; MacQueen et al., 2020), such as the data from the SuperSour strategy with 15 concurrent sweet orange rootstock trials and 350 hybrid rootstocks. We plan to use this multi-trial, multi-year data to map rootstock influence on tree survival, tree size, tree canopy health, disease tolerance, fruit productivity, and a range of important fruit quality traits, and then create easily applicable molecular markers to aid selection of future superior hybrid rootstocks in the breeding program. Selection using molecular markers associated with multiple important rootstock traits (such as survival and productivity) will allow field trials to focus on a pre-selected group of hybrids with the best genetic potential to be outstanding.

A CONTINUUM TO COMMERCIAL USE

The SuperSour strategy makes use of more diverse and elite germplasm, eliminates preselection for apomictic seed production, integrates rapid vegetative propagation for early testing of new hybrids, and evaluates very large numbers of new hybrid rootstocks in multiple concurrent trials to allow relatively rapid comparisons of large numbers of new hybrids for good rootstock traits. Preliminary evaluation of results from two of these trials indicates that results from the individual trials and cross-comparisons between trials can be used to effectively identify positive and negative attributes for each hybrid clone and to select the most superior rootstocks for commercial release. Results from the trials will be used to evaluate the usefulness of several possible selection indices, which may more clearly identify rootstocks with the best combination of important traits. This same information can be used for development of genetic maps and molecular markers to speed the next cycle of rootstock development.

While the SuperSour strategy empowers more rapid development of new rootstocks with more diverse genetics, it is not the end of the rootstock development process. Knowledge about rootstock tolerance or resistance to a particular abiotic stress (flooding, drought, salinity, or cold) and biotic stress (nematodes, CTV, *Phytophthora*, or CLas) is of great value, as it helps to define critical limitations to conditions under which the new rootstock should be used. Within the USDA program, this focused testing is conducted on the most outstanding of the candidates as they reach the end of SuperSour strategy field trials. Rootstocks with individual faults may be of commercial value, as long as the limit of that fault is understood. Similarly, the graft compatibility of the new rootstocks with the broad range of commercially important scions needs to be assessed, to avoid unanticipated graft-incompatibility in the commercial nursery or in the field. Within the SuperSour strategy, focused trials containing the new rootstocks grafted with the major scion cultivar types are conducted to complement the field trials with sweet orange scion. It may also be noted that superior new rootstock cultivars that cannot be uniformly propagated by seed will be much slower to be used in new citrus growing regions because of the extensive inter-region quarantine regulations that limit movement of vegetative plant material (Lavagi-Craddock et al., 2021).

The SuperSour strategy allows the development of new rootstocks in as little as 8 years from cross to release. However, additional time is needed to fully evaluate nursery traits, tolerance to abiotic and biotic stresses, and compatibility with a range of different scions. Depending on the production situation, commercial use with sweet orange in the tested environments would be appropriate immediately, while commercial use with other scions or in other situations may await further trials and testing. The intention of the SuperSour strategy is to create a cycle that repeatedly creates promising new hybrids from elite and exotic germplasm, establishes new trials, conducts specialized testing, and then uses data about those hybrids in the trials and testing to identify superior rootstocks for commercial release, and guide the next cycle of rootstock breeding.

AUTHOR CONTRIBUTIONS

KB conceptualized the work, wrote the first draft of the manuscript, and critically edited the manuscript for publication. GM and UA conducted important portions of the work described in the manuscript and edited and added to the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

KB and UA were supported by the National Institutes of Food and Agriculture (NIFA) through grant 2017-70016-26328. KB and GM were supported by the Citrus Research and Development Foundation through grants 508 and 15-002. KB, GM, and UA were supported by the Citrus Research and Development Foundation through grant 18-004. KB and UA were supported by the Citrus Research and Development Foundation through grant 18-028. UA was supported by the UF/IFAS Citrus Research Initiative and USDA NIFA Hatch project No. 1011775.

ACKNOWLEDGMENTS

The authors thank the Florida Citrus Research Foundation and FDACS for important contributions to the effort of developing new USDA citrus rootstocks.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.741009/full#supplementary-material

REFERENCES

Albrecht, U., Bodaghi, S., Meyering, B., and Bowman, K. D. (2020). Influence of rootstock propagation method on traits of grafted sweet orange trees. *HortScience* 55, 729–737. doi: 10.21273/HORTSCI14928-20

Albrecht, U., Bordas, M., Lamb, B., Meyering, B., and Bowman, K. D. (2017). Influence of propagation method on root architecture and other traits of young citrus rootstock plants. *HortScience* 52, 1569–1576. doi: 10.21273/HORTSCI12320-17

Albrecht, U., and Bowman, K. D. (2004). Growth comparison of citrus rootstocks after artificial infection with Phytophthora. *Proc. Fla. State Hort. Soc.* 117, 156–160.

Albrecht, U., and Bowman, K. D. (2007). Inducible proteins in citrus rootstocks with different tolerance towards the root rot pathogen *Phytophthora palmivora*. *J. Phytopathol.* 155, 606–615.

Albrecht, U., and Bowman, K. D. (2011). Tolerance of the trifoliate citrus hybrid US-897 (*Citrus reticulata x Poncirus trifoliata*) to huanglongbing. *HortScience* 46, 16–22.

Albrecht, U., and Bowman, K. D. (2012). Tolerance of trifoliate citrus rootstock hybrids to *Candidatus* Liberibacter asiaticus. *Sci. Hortic.* 147, 71–80.

Albrecht, U., and Bowman, K. D. (2019). Reciprocal influence of rootstock and scion citrus cultivars challenged with *Ca.* Liberibacter asiaticus. *Sci. Hortic.* 254, 133–144.

Allard, R. W. (1960). *Principles of Plant Breeding*. New York: John Wiley and Sons.

Allario, T., Brumos, J., Colmenero-Flores, J. M., Iglesias, D. J., Pina, J. A., Navarro, L., et al. (2013). Tetraploid Rangpur lime rootstock increases drought tolerance via enhanced constitutive root abscisic acid production. *Plant Cell Environ.* 36, 856–868. doi: 10.1111/pce.12021

Alvarez, S., Rohrig, E., Solís, D., and Thomas, M. (2016). Citrus greening disease (Huanglongbing) in Florida: economic impact, management and the potential for biological control. *Agric. Res.* 5, 109–118. doi: 10.1007/s40003-016-0204-z

Barcelos-Bisi, R., Albrecht, U., and Bowman, K. D. (2020). Seed and seedling nursery characteristics for 10 USDA citrus rootstocks. *HortScience* 55, 528–532. doi: 10.21273/HORTSCI14598-19

Bar-Joseph, M., Marcus, R., and Lee, R. F. (1989). The continuous challenge of citrus tristeza virus control. *Annu. Rev. Phytopathol.* 27, 291–316.

Barrett, H. C., and Hutchison, D. J. (1978). Spontaneous tetraploidy in apomictic seedlings of Citrus. *Econ. Bot.* 32, 27–45. doi: 10.1007/BF02906727

Bassanezi, R. B., Lopes, S. A., de Miranda, M. P., Wulff, N. A., Volpe, H. X. L., Ayres, J. A., et al. (2020). Overview of citrus huanglongbing spread and management strategies in Brazil. *Trop. Plant Pathol.* 45, 251–264. doi: 10.1007/s40858-020-00343-y

Benyon, L., Stover, E., Bowman, K. D., Niedz, R., Shatters, R. J. Jr., Zale, J., et al. (2013). GUS expression driven by constitutive and phloem-specific promoters in citrus hybrid US-802. *Vitro Cell Dev. Biol. Plant* 49, 255–265.

Bitters, W. P. (1986). *Citrus Rootstocks: Their Characters and Reactions*. Riverside, CA: UC Riverside Science Library.

Bitters, W. P., Brusca, J. A., and Cole, D. A. (1964). The search for new citrus rootstocks. *Citrograph* 49, 443–448.

Bitters, W. P., Cole, D. A., and Brusca, J. A. (1969). The citrus relatives as citrus rootstocks. *Proc. First Int. Citrus Sympos.* 1, 411–415.

Bove, J. M. (2006). Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* 88, 7–37.

Bowman, K. D., Albano, J. P., and Graham, J. H. (2002). Greenhouse testing of rootstocks for resistance to Phytophthora species in flatwoods soil. *Proc. Fla State Hort. Soc.* 115, 10–13.

Bowman, K. D., and Albrecht, U. (2015). Comparison of gene expression changes in susceptible, tolerant, and resistant hosts in response to infection with Citrus tristeza virus and huanglongbing. *J. Citrus Pathol.* 2, 1–6.

Bowman, K. D., and Albrecht, U. (2017). Efficient propagation of citrus rootstocks by stem cuttings. *Sci. Hort.* 225, 681–688. doi: 10.1016/j.scienta.2017.07.049

Bowman, K. D., and Albrecht, U. (2020). Rootstock influences on health and growth following *Candidatus* liberibacter asiaticus infection in young sweet orange trees. *Agronomy* 10:1907. doi: 10.3390/agronomy10121907

Bowman, K. D., and Garnsey, S. M. (2001). A comparison of five sour orange rootstocks and their response to Citrus tristeza virus. *Proc. Fla State Hort. Soc.* 114, 73–77.

Bowman, K. D., Gmitter, F. G., and Hu, X. (1995). Relationships of seed size and shape with polyembryony and the zygotic or nucellar origin of Citrus spp. seedlings. *HortScience* 30, 1279–1282.

Bowman, K. D., Gmitter, F. G., Moore, G. A., and Rouseff, R. L. (1991). Citrus-fruit sector chimeras as a genetic resource for cultivar improvement. *J. Am. Soc. Hort. Sci.* 116, 888–893.

Bowman, K. D., Graham, J. H., and Adair, R. C. (2003). Young tree growth in a flatwoods rootstock trial with Diaprepes weevil and Phytophthora diseases. *Proc. Fla. State Hort. Soc.* 116, 249–251.

Bowman, K. D., Hartman, R. D., Lamb, A. E., and Wutscher, H. K. (1997). Enhancing development of improved rootstocks by tissue culture propagation and field performance of selected rootstocks. *Proc. Fla State Hort. Soc.* 110, 10–13.

Bowman, K. D., and Joubert, J. (2020). "Citrus Rootstocks," in *The Genus Citrus*, 1st Edn, eds M. Talon, M. Caruso, and F. G. Gmitter (Cambridge, UK: Elsevier), 105–127.

Bowman, K. D., and McCollum, G. (2015). Five new citrus rootstocks with improved tolerance to huanglongbing. *HortScience* 50, 1731–1734. doi: 10.21273/HORTSCI.50.11.1731

Bowman, K. D., Faulkner, L., and Kesinger, M. (2016a). New citrus rootstocks released by USDA 2001-2010: field performance and nursery characteristics. *HortScience* 51, 1208–1214. doi: 10.21273/HORTSCI10970-16

Bowman, K. D., McCollum, G., and Albrecht, U. (2016b). Performance of 'Valencia' orange (*Citrus sinensis* [L] Osbeck) on 17 rootstocks in a trial severely affected by huanglongbing. *Sci. Hortic.* 201, 355–361. doi: 10.1016/j.scienta.2016.01.019

Bowman, K. D., Shapiro, J. P., and Lapointe, S. L. (2001). Sources of resistance to diaprepes weevil in subfamily aurantiodeae, rutaceae. *HortScience* 36, 332–336.

Cameron, J. W., and Soost, R. K. (1979). Sexual and nucellar embryony in F1 hybrids and advanced crosses of Citrus with Poncirus. *J. Am. Soc. Hort. Sci.* 104, 408–410.

Cameron, J. W., and Soost, R. K. (1986). C35 and C32: Citrange rootstocks for citrus. *HortScience* 21, 157–158.

Cantuarias-Avilés, T., Mourão Filho, F. D. A. A., Stuchi, E. S., da Silva, S. R., and Espinoza-Núñez, E. (2010). Tree performance and fruit yield and quality of 'Okitsu' Satsuma mandarin grafted on 12 rootstocks. *Sci. Hortic.* 123, 318–322. doi: 10.1016/j.scienta.2009.09.020

Carbonell-Caballero, J., Alonso, R., Ibañez, V., Terol, J., Talon, M., and Dopazo, J. (2015). A Phylogenetic analysis of 34 chloroplast genomes elucidates the relationships between wild and domestic species within the genus *Citrus*. *Mol. Biol. Evol.* 32, 2015–2035. doi: 10.1093/molbev/msv082

Carimi, F., and De Pasquale, F. (2003). "Micropropagation of citrus," in *Micropropagation of Woody Trees and Fruits*, eds S. Mohan Jain and I. Katsuaki (Dordrecht: Springer), 589–619.

Caruso, M., Continella, A., Modica, G., Pannitteri, C., Russo, R., Salonia, F., et al. (2020). Rootstocks influence yield precocity, productivity, and pre-harvest fruit drop of Mandared pigmented mandarin. *Agronomy* 10:1305. doi: 10.3390/agronomy10091305

Carvalho, L. M., Carvalho, H. W. L., Barros, I., Martins, C. R., Soares Filho, W. S., Girardi, E. A., et al. (2019). New scion-rootstock combinations for diversification of sweet orange orchards in tropical hardsetting soils. *Sci. Hortic.* 243, 169–176. doi: 10.1016/j.scienta.2018.07.032

Castle, W. S. (1987). "Citrus rootstocks," in *Rootstocks for Fruit Crops*, eds R. Rom and R. Carlson (New York, NY: John Wiley and Sons), 361–399.

Castle, W. S. (2010). A career perspective on citrus rootstocks, their development, and commercialization. *HortScience* 45, 11–15. doi: 10.21273/HORTSCI.45.1.11

Castle, W. S., Bowman, K. D., Baldwin, J. C., Grosser, J. W., and Gmitter, F. G. (2011). Rootstocks affect tree growth, yield, and juice quality of 'Marsh' grapefruit. *HortScience* 46, 841–848. doi: 10.21273/HORTSCI.46.6.841

Castle, W. S., Wutscher, H., Youtsey, C., and Pelosi, R. R. (1988). Citrumelos as rootstocks for Florida citrus. *Proc. Fla. State Hort. Soc.* 101, 28–33.

Cervera, M., Pina, J. A., Juarez, J., Navarro, L., and Pena, L. (1998). Agrobacterium-mediated transformation of citrange: factors affecting transformation and regeneration. *Plant Cell Rep.* 18, 271–278. doi: 10.1007/s002990050570

Continella, A., Pannitteri, C., La Malfa, S., Legua, P., Distefano, G., Nicolosi, E., et al. (2018). Influence of different rootstocks on yield precocity and fruit quality of 'Tarocco Scirè' pigmented sweet orange. *Sci. Hortic.* 230, 62–67. doi: 10.1016/j.scienta.2017.11.006

Cooper, W. C., Reece, P. C., and Furr, J. R. (1962). Citrus breeding in Florida – Past, present, and future. *Proc. Fla State Hort. Soc.* 75, 5–12.

Costa, D. P., Ribeiro, L. D. O., Coelho, M. A., Ledo, C. A. D. S., Stuchi, E. S., Girardi, E. A., et al. (2020a). Nonparametric indices for the selection of hybrid citrus as rootstocks grafted with 'Valência' sweet orange. *Pesquisa Agropecuária Brasileira* 55:e01592. doi: 10.1590/S1678-3921

Costa, D. P., Stuchi, E. S., Girardi, E. A., Gesteira, A. S., Coelho Filho, M. A., Ledo, C. A. S., et al. (2020b). Hybrid rootstocks for Valencia sweet orange in rainfed cultivation under tropical savannah climate. *J. Agric. Sci.* 12, 40–55. doi: 10.5539/jas.v12n11p40

Costa, D. P., Stuchi, E. S., Girardi, E. A., Moreira, A. S., Gesteira, A. S., Coelho Filho, M. A., et al. (2021). Less is more: A hard way to get potential dwarfing hybrid rootstocks for Valencia sweet orange. *Agriculture* 11:354. doi: 10.3390/agriculture11040354

Cruz, M. A. D., Neves, C. S. V. J., Carvalho, D. U. D., Colombo, R. C., Leite Júnior, R. P., and Tazima, Z. H. (2019). 'Navelina'sweet orange trees on five rootstocks in Northern Parana state, Brazil. *Revista Brasileira de Fruticul.* 41:3.

Curtolo, M., de Souza Pacheco, I., Boava, L. P., Takita, M. A., Granato, L. M., Galdeano, D. M., et al. (2020). Wide-ranging transcriptomic analysis of *Poncirus trifoliata, Citrus sunki, Citrus sinensis* and contrasting hybrids reveals HLB tolerance mechanisms. *Sci. Rep.* 10, 1–14. doi: 10.1038/s41598-020-77840-2

Dala-Paula, B. M., Plotto, A., Bai, J., Manthey, J. A., Baldwin, E. A., Ferrarezi, R. S., et al. (2019). Effect of huanglongbing or greening disease on orange juice quality, a review. *Front. Plant Sci.* 9:1976. doi: 10.3389/fpls.2018.01976

Dambier, D., Benyahia, H., Pensabene-Bellavia, G., Aka Kaçar, Y., Froelicher, Y., Belfalah, Z., et al. (2011). Somatic hybridization for citrus rootstock breeding: an effective tool to solve some important issues of the Mediterranean citrus industry. *Plant Cell Rep.* 30, 883–900. doi: 10.1007/s00299-010-1000-z

de Carvalho, D. U., Neves, C. S. V. J., da Cruz, M. A., Colombo, R. C., Yada, I. F. U., Junior, R. P. L., et al. (2021). Performance of 'Salustiana'sweet orange on different rootstocks under Brazilian subtropical conditions. *Sci. Hortic.* 287:110226. doi: 10.1016/j.scienta.2021.110226

Fasahat, P., Rajabi, A., Rad, J. M., and Derera, J. (2016). Principles and utilization of combining ability in plant breeding. *Biom. Biostat. Int J.* 4:85. doi: 10.15406/bbij.2016.04.00085

Ferguson, J., Young, M., and Halvorson, J. (1985). The propagation of citrus rootstocks by stem cuttings. *Proc. Fla. State Hort. Soc.* 98, 39–42.

Forner, J. B., Forner-Giner, M. A., and Alcaide, A. (2003). Forner-Alcaide 5 and Forner-Alcaide 13: two new citrus rootstocks released in Spain. *HortScience* 38, 629–630.

Forner-Giner, M. A., Alcaide, A., Primo-Millo, E., and Forner, J. B. (2003). Performance of 'Navelina'orange on 14 rootstocks in Northern Valencia (Spain). *Sci. Hortic.* 98, 223–232.

Forner-Giner, M. A., Primo-Millo, E., and Forner, J. (2009). Performance of Forner-Alcaide 5 and Forner-Alcaide 13, hybrids of Cleopatra mandarin × *Poncirus trifoliata*, as salinity-tolerant citrus rootstocks. *J. Am. Pomolog. Soc.* 63, 72–80.

Forner-Giner, M. A., Rodriguez-Gamir, J., Martinez-Alcantara, B., Quinones, A., Iglesias, D. J., Primo-Millo, E., et al. (2014). Performance of Navel orange trees grafted onto two new dwarfing rootstocks (Forner-Alcaide 517 and Forner-Alcaide 418). *Sci. Hortic.* 179, 376–387. doi: 10.1016/j.scienta.2014.07.032

Freeman, B., Broadbent, P., and Nicholls, P. (1986). Evaluation of rootstocks for five lemon scions in replant ground. *Aust. J. Exp. Agric.* 26, 751–759.

Frost, H. B., and Soost, R. K. (1968). "Seed reproduction, development of gametes and embryos," in *The Citrus Industry*, Vol. 2, eds W. Reuther, L. D. Batchelor, and H. B. Webber (Berkeley: University of California), 290–324.

Garcia, R., Asins, M. J., Forner, J., and Carbonell, E. A. (1999). Genetic analysis of apomixis in citrus and poncirus by molecular markers. *Theor. Appl. Genet.* 99, 511–518.

Girardi, E. A., Cerqueira, T. S., Cantuarias-Avilés, T. E., Silva, S. R., and Stuchi, E. S. (2017). Sunki mandarin and Swingle citrumelo as rootstocks for rain-fed cultivation of late-season sweet orange selections in northern São Paulo state, Brazil. *Bragantia* 76, 501–511. doi: 10.1590/1678-4499.2016.350

Graham, J., Gottwald, T., and Setamou, M. (2020). Status of huanglongbing (HLB) outbreaks in Florida, California and Texas. *Trop. Plant Pathol.* 45, 265–278. doi: 10.1007/s40858-020-00335-y

Graham, J. H., Bowman, K. D., Bright, D. B., and Adair, R. C. Jr. (2007). Screening citrus rootstock genotypes for tolerance to the Phytophthora–Diaprepes complex under field conditions. *Proc. Fla. State Hort. Soc.* 120, 97–102.

Grosser, J. W., Barthe, G. A., Castle, B., Gmitter, F. G., and Lee, O. (2015). The development of improved tetraploid citrus rootstocks to facilitate advanced production systems and sustainable citriculture in Florida. *Proc. XII Int. Citrus Cong. Int. Soc. Citricul.* 1065, 319–327.

Grosser, J. W., and Gmitter, F. G. (2011). Protoplast fusion for production of tetraploids and triploids: applications for scion and rootstock breeding in citrus. *Plant Cell Tissue Organ Cult.* 104, 343–357.

Grosser, J. W., Gmitter, F. G., and Bowman, K. D. (2020). *New rootstocks in the citrus breeding pipeline. Citrus Industry magazine online (July 15, 2020)*. Newberry, FL: AgNet Media.

Grosser, J. W., Gmitter, F. G., and Castle, W. S. (2016). Breeding citrus rootstocks to mitigate Huanglongbing (HLB, or citrus greening disease). *Acta Hortic.* 1127, 83–88. doi: 10.17660/ActaHortic.2016.1127.15

Grosser, J. W., Jiang, J., Louzada, E. S., Chandler, J. L., and Gmitter, F. G. (1998). Somatic hybridization, an integral component of citrus cultivar improvement: II. Rootstock improvement. *HortScience* 33, 1060–1061.

Grosser, J. W., Medina-Urrutia, V., Ananthakrishnan, G., and Serrano, P. (2004). Building a replacement sour orange rootstock: somatic hybridization of selected mandarin plus pummelo combinations. *J. Am. Soc. Hortic. Sci.* 129, 530–534.

Grosser, J. W., Mourao-Fo, F. A. A., Gmitter, F. G., Louzada, E. S., Jiang, J., Baergen, K., et al. (1996). Allotetraploid hybrids between Citrus and seven related genera produced by somatic hybridization. *Theor. Appl. Genet.* 92, 577–582.

Haroldsen, V. M., Szczerba, M. W., Aktas, H., Lopez-Baltazar, J., Odias, M. J., Chi-Ham, C. L., et al. (2012). Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. *Front. Plant Sci.* 3:39. doi: 10.3389/fpls.2012.00039

Hutchison, D. J. (1974). Swingle citrumelo—A promising rootstock hybrid. *Proc. Fla. State Hort. Soc.* 87, 89–91.

Hutchison, D. J. (1976). Interspecific hybridization in citrus rootstocks. *Fruit Var. J.* 30:34.

Hutchison, D. J. (1985). Rootstock development screening and selection for disease tolerance and horticultural characteristics. *Fruit Var. J.* 39, 21–25.

Ibrahim, A. K., Zhang, L., Niyitanga, S., Afzal, M. Z., Xu, Y., Zhang, L., et al. (2020). Principles and approaches of association mapping in plant breeding. *Trop Plant Biol.* 13, 212–224. doi: 10.1007/s12042-020-09261-4

Jia, H., Orbović, V., and Wang, N. (2019). CRISPR-LbCas12a-mediated modification of citrus. *Plant Biotechnol. J.* 17, 1928–1937. doi: 10.1111/pbi.13109

Jia, H. G., and Wang, N. (2014). Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS One* 9:e93806. doi: 10.1371/journal.pone.0093806

Kepiro, J. L., and Roose, M. L. (2007). "Nucellar embryony," in *Citrus Genetics, Breeding and Biotechnology*, ed. I. A. Khan (Wallingford: CABI), 141–150.

Kepiro, J. L., and Roose, M. L. (2010). AFLP markers closely linked to a major gene essential for nucellar embryony (apomixis) in *Citrus maxima* × *Poncirus trifoliata*. *Tree Genet. Genomes* 6, 1–11.

Koltunow, A. M., Soltys, K., Nito, N., and McClure, S. (1995). Anther, ovule, seed, and nucellar embryo development in *Citrus sinensis* cv. *Valencia. Can. J. Bot.* 73, 1567–1582.

Kunwar, S., Grosser, J., Gmitter, F. G., Castle, W. S., and Albrecht, U. (2021). Field performance of 'Hamlin' orange trees grown on various rootstocks in huanglongbing-endemic conditions. *HortScience* 56, 244–253.

Lapin, W. (1937). Investigation on polyploidy in Citrus. *USSR All Union Sci. Res. Inst.* 1, 1–68.

Lavagi-Craddock, I., Greer, G., Grosser, J., Gmitter, F. G., Bowman, K. D., Stover, E., et al. (2021). Interstate movement of new and licensed citrus varieties. *Citrograph* 12, 60–63.

Lee, A. T. C., Joubert, J., and van Vuuren, S. P. (2009). *"Chapter 6: Rootstock choice," in Integrated Production Guidelines for Export Citrus*, Vol. 1. Mbombela: Citrus Research International (Pty) Ltd.

Li, S., Wu, F., Duan, Y., Singerman, A., and Guan, Z. (2020). Citrus greening: Management strategies and their economic impact. *HortScience* 55, 604–612. doi: 10.21273/HORTSCI14696-19

Lima, R. P., Curtolo, M., Merfa, M. V., Cristofani-Yaly, M., and Machado, M. A. (2018). QTLs and eQTLs mapping related to citrandarins' resistance to citrus gummosis disease. *BMC Genom.* 19:516. doi: 10.1186/s12864-018-4888-2

Llosa, M. J., Bermejo, A., Cano, A., Quiñones, A., and Forner-Giner, M. A. (2009). The citrus rootstocks Cleopatra mandarin, *Poncirus trifoliata*, Forner-Alcaide 5 and Forner-Alcaide 13 vary in susceptibility to iron deficiency chlorosis. *J. Am. Pomolog. Soc.* 63, 160–167.

Long, K., Fraser, L., Bacon, P., and Broadbent, P. (1977). The Benton Citrange: A promising Phytophthora resistant root-stock for citrus trees. *Proc. Int. Soc. Citricul.* 2, 541–544.

Louzada, E. S., del Rio, H. S., Sétamou, M., Watson, J. W., and Swietlik, D. M. (2008). Evaluation of citrus rootstocks for the high pH, calcareous soils of South Texas. *Euphytica* 164, 13–18.

Lucht, J. M. (2015). Public acceptance of plant biotechnology and GM crops. *Viruses* 7, 4254–4281.

MacQueen, A. H., White, J. W., Lee, R., Osorno, J. M., Schmutz, J., Miklas, P. N., et al. (2020). Genetic associations in four decades of multienvironment trials reveal agronomic trait evolution in common bean. *Genetics* 215, 267–284. doi: 10.1534/genetics.120.303038

Marques, L. O. D., Mello-Farias, P., Oliveira, R. P. D., Dini, M., Santos, R. F. D., Malgarim, M. B., et al. (2021). *Nursery performance of potentially promising rootstocks for citriculture in the south of Brazil*. Santa Maria: Ciência Rural, 52.

Martínez-Cuenca, M.-R., Primo-Capella, A., and Forner-Giner, M. A. (2016). *Influence of Rootstock on Citrus Tree Growth: Effects on Photosynthesis and Carbohydrate Distribution, Plant Size, Yield, Fruit Quality, and Dwarfing Genotypes, Plant Growth, Everlon Cid Rigobelo*. London: IntechOpen.

Moreira, C. D., Chase, C. D., Gmitter, F. G., and Grosser, J. W. (2000). Inheritance of organelle genomes in citrus somatic cybrids. *Mol. Breed.* 6, 401–405.

Mylavarapu, R., Harris, W., and Hochmuth, G. (2019). *Agricultural soils of Florida*. Florida: University of Florida IFAS Extension, publication.

Oakey, H., Verbyla, A., Pitchford, W., Cullis, B., and Kuchel, H. (2006). Joint modeling of additive and non-additive genetic line effects in single field trials. *Theor. Appl. Genet.* 113, 809–819.

Ohgawara, T., Kobayashi, S., Ohgawara, E., Uchimiya, H., and Ishii, S. (1985). Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. *Theor. Appl. Genet.* 71, 1–4. doi: 10.1007/BF00278245

Oliveira, T. M., Ben Yahmed, J., Dutra, J., Maserti, B. E., Talon, M., Navarro, L., et al. (2017). Better tolerance to water deficit in doubled diploid 'Carrizo citrange' compared to diploid seedlings is associated with more limited water consumption and better H_2O_2 scavenging. *Acta Physiol. Plantarum* 39:e204.

Ollitrault, P., Germanà, M. A., Froelicher, Y., Cuenca, J., Aleza, P., Morillon, R., et al. (2020). "Ploidy manipulation for citrus breeding, genetics, and genomics," in *The Citrus Genome. Compendium of Plant Genomes*, eds A. Gentile, S. La Malfa, and Z. Deng (Switzerland AG: Springer Nature), doi: 10.1007/978-3-030-15308-3_6

Oustric, J., Morillon, R., Luro, F., Herbette, S., Lourkisti, R., Giannettini, J., et al. (2017). Tetraploid Carrizo citrange rootstock (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) enhances natural chilling stress tolerance of common clementine (*Citrus clementina* Hort. ex Tan). *J. Plant Physiol.* 214, 108–115.

Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., et al. (2017). Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus. *Plant Biotechnol. J.* 15, 1509–1519. doi: 10.1111/pbi.12733

Petri, C., Alburquerque, N., Faize, M., Scorza, R., and Dardick, C. (2018). Current achievements and future directions in genetic engineering of European plum (*Prunus domestica* L.). *Transgenic Res.* 27, 225–240. doi: 10.1007/s11248-018-0072-3

Pokhrel, S., Meyering, B., Bowman, K. D., and Albrecht, U. (2021). Horticultural attributes and root architectures of field-grown 'Valencia' trees grafted on different rootstocks propagated by seed, cuttings, and tissue culture. *HortScience* 56, 163–172.

Poles, L., Licciardello, C., Distefano, G., Nicolosi, E., Gentile, A., and La Malfa, S. (2020). Recent advances of in vitro culture for the application of new breeding techniques in citrus. *Plants* 9:938.

Prakash, S., and Sharma, S. (2018). Micropropagation of citrus rootstock. *J. Pharmacogn. Phytochem.* 7, 1513–1517.

Reforgiato Recupero, G., Russo, G., Recupero, S., Zurru, R., Deidda, B., and Mulas, M. (2009). Horticultural evaluation of new Citrus latipes hybrids as rootstocks for citrus. *HortScience* 44, 595–598.

Reforgiato Recupero, G., and Russo, M. P. (1992). Percents of nucellar embryony in some hybrid populations of *Citrus latipes*. *Proc. Intl. Soc. Citricul.* 1, 130–134.

Rife, T., Graybosch, R., and Poland, J. (2018). Genomic analysis and prediction within a US public collaborative winter wheat regional testing nursery. *Plant Genome* 11, 1–7. doi: 10.3835/plantgenome2018.01.0004

Rodriguez, M., Monter, A., Espinosa, A., Castaneda, C. G., and Velázquez, G. A. (2005). Polyembryony and RAPD markers for identification of zygotic and nucellar seedlings in Citrus. *Agrociencia* 39, 371–383.

Rodriguez-Gamir, J., Primo-Millo, E., Forner, J. B., and Forner-Giner, M. A. (2010). Citrus rootstock responses to water stress. *Scientia Hortic.* 126, 95–102. doi: 10.1016/j.scienta.2010.06.015

Rosson, B. (2020). *Citrus Budwood Annual Report 2019-2020*. Tallahassee, FL: Florida Department of Agriculture and Consumer Services.

Ruiz, M., Pensabene-Bellavia, G., Quinones, A., Garcia-Lor, A., Morillon, R., Ollitrault, P., et al. (2018). Molecular characterization and stress tolerance evaluation of new allotetraploid somatic hybrids between *Carrizo citrange* and *Citrus macrophylla* W. rootstocks. *Front. Plant Sci.* 9:901. doi: 10.3389/fpls.2018.00901

Russo, F., and Reforgiato Recupero, G. (1984). Recent results of some Citrus rootstocks experiments in Italy. *Proc. Intl. Soc. Citriculture* 1, 42–45.

Russo, F., and Torrisi, M. (1951). Il polliploidismo nei citrus autopoliploidi ed allopoliploidi. *Ann. Sper. Agr.* 5, 1041–1062.

Salonia, F., Ciacciulli, A., Poles, L., Pappalardo, H. D., La Malfa, S., and Licciardello, C. (2020). New plant breeding techniques in citrus for the improvement of important agronomic traits. A Review. *Front. Plant Sci.* 11:1234. doi: 10.3389/fpls.2020.01234

Santana-Vieira, D. D. S., Freschi, L., da Hora Almeida, L. A., de Moraes, D. H. S., Neves, D. M., Dos Santos, L. M., et al. (2016). Survival strategies of citrus rootstocks subjected to drought. *Sci. Rep.* 6, 1–12. doi: 10.1038/srep38775

Sen, S., and Dhawan, V. (2010). Development of a highly efficient micropropagation method for the citrus rootstock 'Swingle' citrumelo [*Poncirus trifoliata* (L.) Raf. × *C. paradisi* McFaden]. *Int. J. Fruit Sci.* 10, 65–78.

Singerman, A., Futch, S. H., and Page, B. (2021). Early performance of selected citrus rootstocks grafted with 'Valencia' sweet orange in commercial settings within a citrus greening endemic environment, *HortTechnology* 31, 417–427. doi: 10.21273/HORTTECH04784-20

Song, G., Prieto, H., and Orbovic, V. (2019). *Agrobacterium*-mediated transformation of tree fruit crops: Methods, progress, and challenges. *Front. Plant Sci.* 10:226. doi: 10.3389/fpls.2019.00226

Song, G. Q., Walworth, A. E., and Loescher, W. H. (2015). Grafting of genetically engineered plants. *J. Am. Soc. Hortic. Sci.* 140, 203–213. doi: 10.21273/JASHS.140.3.203

Soratto, T. A. T., Curtolo, M., Marengo, S., Dezotti, A. L., Lima, R. P. M., Gazaffi, R., et al. (2020). QTL and eQTL mapping associated with host response to *Candidatus Liberibacter asiaticus* in citrandarins. *Trop. Plant Pathol.* 45, 626–645. doi: 10.1007/s40858-020-00372-7

Stansly, P. A., Arevalo, H. A., Qureshi, J. A., Jones, M. M., Hendricks, K., Roberts, P. D., et al. (2014). Vector control and foliar nutrition to maintain economic sustainability of bearing citrus in Florida groves affected by huanglongbing. *Pest. Mgt. Sci.* 70, 415–426. doi: 10.1002/ps.3577

Swingle, W. T., and Reece, C. (1967). "The botany of Citrus and its wild relatives," in *The Citrus Industry*, eds W. Reuther, H. J. Webber, and L. D. Batchelor (Berkeley and Los Angeles: University of California Press), 191–430.

Tolkowsky, S. (1938). *Hesperides. A history of the culture and use of citrus fruits*. London: John Bales, Sons & Curnow, Ltd.

Velazquez, K., Aguero, J., Vives, M. C., Aleza, P., Pina, J. A., Moreno, P., et al. (2016). Precocious flowering of juvenile citrus induced by a viral vector based on Citrus leaf blotch virus: a new tool for genetics and breeding. *Plant Biotechnol. J.* 14, 1976–1985. doi: 10.1111/pbi.12555

Verdejo-Lucas, S., Sorribas, F. J., Forner, J. B., and Alcaide, A. (1997). Screening hybrid citrus rootstocks for resistance to *Tylenchulus semipenetrans* Cobb. *HortScience* 32, 1116–1119.

von Broembsen, L. A. (1985). *Citrus rootstocks-the choice you have*. Pretoria, South Africa: South African Co-operative Citrus Exchange.

Wang, L., Chen, S., Peng, A., Xie, Z., He, Y., and Zou, X. (2019). CRISPR/Cas9-mediated editing of CsWRKY22 reduces susceptibility to *Xanthomonas citri* subsp. *citri* in Wanjincheng orange (*Citrus sinensis* (L.) Osbeck). *Plant Biotechnol. Rep.* 13, 501–510. doi: 10.1007/s11816-019-00556-x

Webber, H. J. (1967). "History and development of the Citrus industry," in *The Citrus Industry*, eds W. Reuther, et al. (Oakland, CL: University of California Press), 1–39.

Wu, G. A., Prochnik, S., Jenkins, J., Salse, J., Hellsten, U., Murat, F., et al. (2014). Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. *Nat. Biotechnol.* 32, 656–662.

Wutscher, H. K. (1979). Citrus rootstocks. *Hort. Rev. (Amer. Soc. Hort. Sci.)* 1, 237–269.

Wutscher, H. K., and Bowman, K. D. (1999). Performance of 'Valencia' orange on 21 rootstocks in Central Florida. *HortScience* 34, 622–624.

Xiang, X., Deng, Z.-A., Chen, C.-X., Gmitter, F. G., and Bowman, K. D. (2010). Marker assisted selection in citrus rootstock breeding based on a major gene locus 'Tyr1' controlling citrus nematode resistance. *Agric. Sci. China* 9, 557–567.

Xu, Y., and Crouch, J. H. (2008). Marker-assisted selection in plant breeding: From publications to practice. *Crop Sci.* 48, 391–407.

Zou, X. P., Peng, A. H., Xu, L. Z., Liu, X. F., Lei, T. G., Yao, L. X., et al. (2013). Efficient auto-excision of a selectable marker gene from transgenic citrus by combining the Cre/loxP system and ipt selection. *Plant Cell Rep.* 32, 1601–1613. doi: 10.1007/s00299-013-1470-x

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 19 November 2021
doi: 10.3389/fpls.2021.777078

Determining Seed Viability During Fruit Maturation to Improve Seed Production and Availability of New Citrus Rootstocks

*Deived Uilian de Carvalho[1,2], Daniel A. Boakye[1], Tim Gast[1], Rui Pereira Leite Junior[2] and Fernando Alferez[1]**

[1] Southwest Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Immokalee, FL, United States, [2] Department of Crop Protection, Instituto de Desenvolvimento Rural do Paraná, Londrina, Brazil

In recent years, the pressure for replanting and resetting huanglongbing (HLB or citrus greening) affected citrus groves has led to an inadequate seed supply for the most popular rootstock cultivars in the State of Florida, United States. Early fruit harvesting of citrus rootstock source trees might reduce fruit losses and enhance seed availability, especially in HLB-endemic and hurricane susceptible areas, if the physiological quality of the seeds is adequate. The effects of fruit maturity on seed quality and seedling performance of US-802, US-897, and US-942 citrus rootstocks were investigated for two consecutive growing seasons. The study included the evaluation of seed germination and nursery performance of the citrus rootstock seedlings. The germination test was performed *in vitro*, where seeds were hand-peeled, surface-sterilized and placed in culture tubes containing basal Murashige and Skoog medium. For the emergence test, seeds were sown in seedling trays containing sterilized growing substrate in a greenhouse with controlled-environment conditions. Rootstock fruits from all three varieties harvested in August and September had seeds with higher germination potential, as more than 90% of the seeds generated seedlings. US-942 had more % of emergence than US-802 and US-897, resulting in faster seed germination; in contrast, US-802 had the faster shoot growth rate. Assays on fruit abscission response showed that by August, fruit from all three varieties were responsive to ethylene and abscised, although response varied and was higher in US-942, suggesting the seeds were mature enough. Taken together, our findings indicate that fruits these three rootstocks can be harvested as early as August in contrast to the current procedures without losing germination potential. This will result in an increase in available seeds for nurseries in Florida.

Keywords: growth rate, nursery assessment, rootstocks, seed germination, seed vigor

OPEN ACCESS

Edited by:
Sergio Ruffo Roberto, State University of Londrina, Brazil

Reviewed by:
Fernando Alves De Azevedo, Sylvio Moreira Citriculture Center, Brazil
Ed Stover, Horticultural Research Laboratory, United States Department of Agriculture, Agricultural Research Service (USDA-ARS), United States

***Correspondence:**
Fernando Alferez
alferez@ufl.edu

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

Received: *14 September 2021*
Accepted: *27 October 2021*
Published: *19 November 2021*

Citation:
de Carvalho DU, Boakye DA, Gast T, Leite Junior RP and Alferez F (2021) Determining Seed Viability During Fruit Maturation to Improve Seed Production and Availability of New Citrus Rootstocks. Front. Plant Sci. 12:777078. doi: 10.3389/fpls.2021.777078

INTRODUCTION

Currently, Florida's citriculture faces an unprecedented crisis due to the widespread presence of citrus greening or Huanglongbing (HLB), a disease associated with the phloem-limited bacterium *Candidatus* Liberibacter asiaticus that has become endemic in the State. Citrus trees affected by HLB are characterized by root loss, branch die-back, yellow shoots, blotchy mottle chlorotic patterns on

leaves, and off-tasting and malformed fruit. HLB infection inhibits root growth, reduces nutrient uptake, and promotes leaf and fruit drop, and whole tree decline that is often lethal (Pustika et al., 2008; Etxeberria et al., 2009). HLB also plays a major role in several aspects of citrus production, including the choice of the citrus rootstock. Replanting HLB-affected trees with disease-free nursery trees may require rootstocks resistant to soil diseases as *Phytophthora* gummosis, in order to guarantee the reestablishment of the grove (Graham et al., 2013).

As mature trees decline due to HLB, there is an increasing demand of citrus rootstocks for replanting. Under this pressure for replanting and resetting HLB-affected groves, there is a clear concern among citrus nursery operators and growers on seed availability of highly demanded rootstocks in Florida. Currently, seed propagation remains the preferred method of rootstock production in commercial citrus nurseries (Albrecht et al., 2020; Pokhrel et al., 2021), in part because there is no evidence on the vertical transmission of viable cells of the HLB pathogen "*Candidatus* Liberibacter spp." through seeds to citrus seedlings (Belasque Júnior et al., 2009; Bagio et al., 2020), and also because the extended belief that different root architectures arising from vegetative propagation will result in trees of inferior quality as compared to those from seeds (Albrecht et al., 2020); as a result, over 80% of the licensed citrus nurseries prefer uniform liners from seeds rather than from tissue culture sources (Chaires, 2017). In any case, vigorous seeds are necessary to produce strong rootstocks. Seed vigor depends on seed maturation and can also be influenced by the extent of cold storage (Carvalho and Silva, 2013). In general, the more mature a seed, the longer it can be stored.

Since rootstock fruits are not edible and/or commercially exploited, little to no attention has been paid to fruit development in rootstock seed cultivars, and as a result, the minimum fruit maturation stage at which citrus rootstock seeds can germinate is unknown. In addition, natural calamities such as tropical storms and hurricanes may affect the number of seeds annually available in Florida (Albrigo et al., 2005; Alferez and Bordas, 2019). The Atlantic hurricane season peaks around September 10th, whereas historically, rootstock fruit harvesting for seed extraction has been performed between October and December. This has resulted in fruit loss and reduction in seed yield due to fruit drop caused by high-speed winds. For instance, Hurricane Irma on September 10th, 2017, severely affected the ability of the industry in Florida to produce adequate number of seeds necessary to satisfy nurseries needs that year. In the hardest hit areas, there was massive fruit drop, especially from the US-802, US-812, US-897, and US-942 rootstock varieties (Alferez, 2018). Seed availability from the SWFREC Immokalee, USDA Fort Pierce, and Whitmore Foundation in Leesburg, the main certified seed suppliers in Florida, decreased by 66% as compared to the previous season, resulting in a shortage in seed availability. This subsequently affected the whole supply chain leading to a reduction in replanting. In this scenario, it will be advantageous to know in advance when the fruit contains viable seeds. This would allow making informed decisions on when to harvest the fruit of each variety, facilitating to work around the peak of the hurricane season.

The information currently available on the effect of fruit age on physiological responses of seedlings that germinated from seed obtained from fruit at different time of the season is very limited (Orbovic et al., 2011, 2013). Since vigorous seed germination and establishment of seedlings with well-defined root growth are necessary for the development of a healthy nursery stock, it is imperative that healthy disease-free seeds with good vigor and viability are utilized to establish a nursery population that can in turn result in vigorous scion growth and rapid establishment in the field. For this it is necessary to know when a seed is mature enough to germinate and with good storability potential. Citrus rootstock fruit, as any other citrus fruit, naturally develop their abscission potential as they mature, i.e., when seeds are able to germinate, as abscission is a natural mechanism for seed dispersion. If harvesting is delayed, this results in a decrease in yield, as the fruit naturally starts to drop.

For all the above reasons it would be advantageous for the citrus nursery industry to find markers of seed maturity that allow harvesting the fruit from these rootstocks when the seed is able to germinate. In this paper we report the evaluation of several candidate markers for seed viability, including fruit color, size, and abscission capacity. Then, we study seed vigor as related to maturation stage. We show that sensitivity to abscission induction is a good marker for seed viability, allowing advancing fruit harvesting significantly without loss of seed vigor, and resulting in better yield of seeds of highly demanded citrus rootstocks in Florida.

MATERIALS AND METHODS

Plant Material

Citrus hybrid rootstock trees from three different varieties planted at the Southwest Florida Research and Education Center, Immokalee, FL, United States, were used in the study as source of fruits and seeds. Fruit samples were collected from eight to 10-year-old grafted seed trees of US-802 (27 trees), US-897 (26 trees), and US-942 (27 trees) for the 2018 (mid-May, June, July, August, and September), 2019 (mid-December), and 2020 (mid-July, August, and September) cropping seasons and immediately transported to the laboratory for further analysis. In 2018, four replicates of 10-fruit sample were randomly selected at 1–2 m height from all rootstock variety trees at each evaluated period for the *in vitro* fruit abscission assay. Later, a batch of 200-fruit were randomly selected at 1–2 m height from all evaluated rootstock variety trees and divided into four replicates of 50 fruits at each harvest date for the 2019 and 2020 cropping seasons, to characterize the rootstock fruits and seeds and to evaluate the seed germination and seedling emergence performance. US-802 rootstock is the result of a cross of "Siamese" pummelo [*Citrus maxima* (Burn.) Merr.] × "Gotha Road" trifoliate orange [*Poncirus trifoliata* (L.) Raf.] while "US-897" originated from a cross of "Cleopatra" mandarin [*Citrus reshni* (Hort.) ex Tan.] × "Flying Dragon" trifoliate orange [*P. trifoliata* (L.) Raf.], both were officially released in 2007 by the Agricultural Research Service (ARS) of the U.S. Department of Agriculture (USDA). Later, in 2010, the "US-942" rootstock was also released.

This rootstock selection was originated from a cross of "Sunki" mandarin [*Citrus sunki* (Hort.) ex Tan.] × "Flying Dragon" trifoliate orange [*P. trifoliata* (L.) Raf.]. These three are among the most sought-after rootstocks in Florida due to their relative novelty and better performance under endemic HLB conditions.

Fruit Characterization

Four replicates of 10 fruits per rootstock were characterized according to their physical parameters at each fruit harvest date. Fruit were weighed (g) using a semi-analytical scale (Radwag, PS 1000.R2, Radom, Poland) and the fruit length (mm) and diameter (mm) were assessed with a digital caliper (Fowler High Precision, Inc., 54-101-150-2, Newton, MA, United States). The fruit shape was determined based on the length/diameter ratio. The fruit color development was monitored during the evaluated period using a portable colorimeter (Konica Minolta, CR-400, Tokyo, Japan). For this, four replicates of five fruit per rootstock were measured and three different readings were obtained along the equatorial circumference of each fruit.

The CIE $L^*a^*b^*$ color scale was adopted (McGuire, 1992), and the citrus color index (CCI) was calculated according to Jiménez-Cuesta et al. (1981):

$$CCI = \frac{1000 \times a^*}{L^* \times b^*}$$

where, *CCI* = citrus color index, a^* = red-green color value, b^* = yellow-blue color value, L^* = lightness.

The CCI is a comprehensive indicator for color impression with positive values for red, negative values for blue-green, and 0 for an intermediate mixture of red, yellow, and blue-green (Zhou et al., 2010). Lightness (L^*) value ranges from 0 to 100 in which higher values indicate lighter color intensity (McGuire, 1992).

Seed Extraction and Characterization

Seeds were hand-extracted from each rootstock fruits, washed under distilled water, and air-dried at room-temperature for 24 h. The number of filled seeds per fruit was counted using four replicates of 10-fruit per rootstock, collapsed and aborted seeds were not included. Seed length and width were measured for each rootstock using a digital caliper (Fowler High Precision, Inc., 54-101-150-2, Newton, MA, United States), and the seed weight was assessed using an analytical scale (Radwag, AS 60/220.R2, Radom, Poland). All seed measurements were based on four replicates of 100-seed sample.

In vitro Seed Germination Evaluation

Seeds from all three rootstocks were individually peeled, standardized for the moisture content for 24 h under controlled-environment conditions (10°C, 100% RH, and dark), surface-sterilized for 20 min in a solution with 5.0% (v/v) sodium hypochlorite (Clorox Co., Oakland, CA, United States) and 0.01% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO, United States), rinsed three times in distilled sterile water, and then sown. The germination test was performed *in vitro* in a complete randomized design using four replicates of 15 peeled seeds extracted from a 50-fruit sample for each harvest date and rootstock, as previously described. One sterile seed was individually placed per culture tube (25 × 150 mm) filled with 18 mL of MS (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose (w/v) and 0.7% agar (w/v) (Murashige and Skoog Basal Medium, M9274, Sigma-Aldrich, St. Louis, MO, United States), previously adjusted to pH of 5.8 and autoclaved at 121°C for 15 min. After sown, the culture tubes were sealed and maintained in growth chamber (Conviron Ltd., CMP6010, Winnipeg, MB, Canada) at constant temperature of 25 ± 1°C and dark for 30 days. Measurements were taken daily after root protrusion in order to calculate the total percentage of germination, root growth rate (RGR), shoot growth rate (SGR), and germination speed index (GSI) (germination rate) that measures the speed of germination and quantifies the seed vigor based on a time-weighted cumulative germination (Brown and Mayer, 1986). The percentage of multiple seedlings per seed (polyembryony) was quantified at the end of the germination evaluation. To calculate the root and SGR we used the following formula:

$$Growth\ rate = \frac{|a_2 - a_1| + |a_3 - a_2| + \cdots + |a_n - a_{n-1}|}{\mathrm{n} - 1}$$

where a_1, a_2,..., $a_{(n)}$, $a_{(n-1)}$ = root or shoot length at corresponding day; and n = number of days. Growth rate was expressed in mm day^{-1}.

The GSI was calculated based on the mathematical expression proposed by Maguire (1962):

$$GSI = \frac{g_1}{n_1} + \frac{g_2}{n_2} + \cdots + \frac{g_n}{n_n}$$

where GSI = germination speed index; g_1, g_2,... g_n = number of germinated seed recorded at the first count, second count,... and last count; n_1, n_2,... n_n = number of days seed have been sown at the first, second,... and last count.

Seedling Emergence Evaluation

Nursery performance assessment of citrus rootstock seedlings was also evaluated using standard nursery methods. The emergence study was set in a complete randomized design using four replicates of 48 seeds extracted from a 50-fruit sample for each harvest date and rootstock, as previously described. One seed per tray cell was planted at 0.5 cm depth into seedling trays of 16.8 × 35.6 × 61.0 cm with 96 cells (Stuewe and Sons, Inc., FT96-7, Tangent, OR, United States) containing sterilized growing medium composed by sphagnum peat moss (Premier Horticulture, Inc., Pro-Mix HP Mycorrhizae, Quakertown, PA, United States). The trays were set on benches inside a controlled-environment greenhouse at the Southwest Florida Research and Education Center (Immokalee, FL, United States) and irrigated daily. The number of emerging seedlings was monitored daily to determine the number of days required to emerge 50% of the population. After 60 days of planting, when seeds ceased emerging, the total percentage of emerged seedlings was determined.

FIGURE 1 | The rootstock seed source plantings at UF-IFAS Southwest Florida Research and Education Center in the aftermath of Hurricane Irma on September 14th, 2017. Note how virtually all rootstock seed-producing fruit from US-802, US-897, and US-942 was lost (Adapted from Alferez et al., 2021a).

TABLE 1 | Seed production of three commercial citrus rootstocks in Florida during two consecutive years, 2016 (no hurricane incidence) and 2017 (hurricane incidence).

Seed Source	Rootstock	2016			2017		
		Extraction	Boxes of fruits	Seed (liter)	Extraction	Boxes of fruits	Seed (liter)
SWFREC[1] (Immokalee, FL, United States)	US-802	December	40	72	October	15	41
	US-897	December	38	84	October	16	60
	US-942	December	18	28	October	4	18
USDA HRL[2] (Ft. Pierce, FL, United States)	US-802	December	13	23	November	6	10
	US-897	December	18	40	November	9	20
	US-942	December	10	16	November	4	10
A.H. Whitmore Foundation (Leesburg, FL, United States)	US-802	December	27	48	November	6	10
	US-897	December	23	51	November	6	14
	US-942	December	10	16	November	5	10
Total (All locations)	US-802	–	80	143	–	27	61
	US-897	–	79	175	–	31	94
	US-942	–	38	60	–	13	38

[1] *Southwest Florida Research and Education Center.*
[2] *U.S. Department of Agriculture, Horticultural Research Laboratory.*

In vitro Fruit Abscission Assay

Additionally, four replicates of 10 fruits per rootstock and treatment, containing the stem (2 cm), were harvested at each harvest date and immediately transported to the lab. A disposable plastic 3 mL Pasteur pipette was attached to the stem and the union sealed with parafilm to prevent leakage. Treatment solutions were applied through the pipette by cutting the upper portion of the pipette. Treatments were water (control) and 1-aminocyclopropane-l-carboxylic acid (ACC; 0.1 mM), as previously described (Merelo et al., 2017). Abscission rate was monitored daily.

Statistical Analysis

The experimental design was completely randomized with a factorial arrangement [three treatments (rootstocks) × four seed extraction periods] replicated four times. The fruit characterization was performed using 10-fruit sample per replicate while 100-seed sample was taken per replicate for seed characterization. The evaluation of seed germination was based on 15-seed sample per replicate and 48-seed sample for the seedling emergence test. These data were submitted to normality and homogeneity of variances tests before analysis of variance (ANOVA) using the ExpDes package (Husson et al., 2017) in *R*

TABLE 2 | Fruit characterization of three citrus rootstocks harvested during different period at the Southwest Florida Research and Education Center, Immokalee, FL, United States (mean value ± standard deviation).

Source of variance	Fruit weight (g)			
Rootstock	**July**	**August**	**September**	**December**
US-802	178 ± 2.88 Da[1]	194 ± 4.78 Ca	249 ± 2.78 Aa	229 ± 3.81 Ba
US-897	29 ± 1.30 Cb	34 ± 2.40 Bb	41 ± 2.29 Ab	32 ± 2.34 BCc
US-942	25 ± 0.94 Db	31 ± 1.13 Cb	43 ± 2.83 Bb	50 ± 1.31 Ab
CV (%)	2.79			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	***			
	Fruit length (mm)			
US-802	69.4 ± 1.20 Ba	69.7 ± 1.41 Ba	75.4 ± 1.62 Aa	74.3 ± 2.15 Aa
US-897	33.2 ± 0.55 Bb	35.1 ± 0.65 Bb	37.9 ± 0.90 Ac	35.0 ± 0.81 Bc
US-942	33.7 ± 0.49 Bb	36.1 ± 0.57 Bb	40.6 ± 1.08 Ab	41.3 ± 2.14 Ab
CV (%)	2.62			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	***			
	Fruit diameter (mm)			
US-802	76.0 ± 0.67 Ba	76.7 ± 1.22 Ba	82.7 ± 1.08 Aa	78.1 ± 2.71 Ba
US-897	39.4 ± 0.75 Bb	41.4 ± 1.08 ABb	43.5 ± 0.24 Ab	40.7 ± 1.28 Bc
US-942	36.2 ± 0.55 Dc	39.2 ± 0.56 Cc	43.5 ± 0.98 ABb	45.8 ± 1.16 Ab
CV (%)	2.22			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	***			
	Fruit shape (length/diameter)			
US-802	0.91 ± 0.01 Ba	0.91 ± 0.01 Ba	0.91 ± 0.01 Ba	0.95 ± 0.01 Aa
US-897	0.84 ± 0.01 Ab	0.85 ± 0.01 Ab	0.87 ± 0.01 Ab	0.86 ± 0.01 Ac
US-942	0.93 ± 0.01 Aa	0.92 ± 0.01 ABa	0.93 ± 0.01 Aa	0.90 ± 0.03 Bb
CV (%)	1.64			
Rootstocks	***			
Harvest	ns			
Rootstock × Harvest	***			

[1]*Means followed by the same capital and lowercase letter in the row and column, respectively, do not significantly differ according to the Tukey's test ($P \leq 0.05$). Significant level: ***$P \leq 0.001$; ns, non-significant.*

v. 4.0.2 (The *R* Foundation for Statistical Computing, Vienna, Austria). Means were compared by Tukey's test and taken to be significantly different at $P \leq 0.05$. The correlation matrices were created for each citrus rootstocks based on the studied traits using the FactoMineR (Lê et al., 2008) and corrplot (Wei et al., 2017) *R* packages. Fruit abscission data from *in vitro* assays was expressed as mean ± std and was subjected to analysis of variance for means separation.

RESULTS AND DISCUSSION

Seed Production

On September 10th of 2017, hurricane Irma crossed the Florida Peninsula from South to North, affecting the seed production of major commercial citrus rootstocks due to tree severe damage and intense fruit drop (**Figure 1**). Seed availability from the three main registered rootstock seed sources in Florida, Southwest Florida Research and Education Center (Immokalee, FL, United States), U.S. Department of Agriculture (Ft. Pierce, FL, United States), and A.H. Whitmore Foundation Farm (Leesburg, FL, United States) decreased by 66% as compared to previous season, resulting in a shortage in seed availability (**Table 1**).

Fruit and Seed Characterization

Significant interaction between rootstock and time of harvest was observed for fruits and seeds (**Tables 2**, **3**). In all three rootstocks studied growth pattern was similar, and typically sigmoidal, as described elsewhere for citrus (Agustí and Primo-Millo, 2020). In September 2020, fruits had the highest weight and size. These values were similar or even higher than those observed in December 2019 when the fruits were fully

TABLE 3 | Characterization of three citrus rootstock seeds extracted during different period at the Southwest Florida Research and Education Center, Immokalee, FL, United States (mean value ± standard deviation).

Source of variance	Seeds per fruit			
Rootstock	**July**	**August**	**September**	**December**
US-802	46 ± 2.59 Ba	46 ± 1.12 Ba	50 ± 1.28 Aa	33 ± 1.53 Ca
US-897	19 ± 1.11 Ab	16 ± 2.23 Bb	19 ± 0.84 Ab	17 ± 1.37 ABb
US-942	15 ± 0.88 Ac	13 ± 1.76 ABc	13 ± 1.32 ABc	12 ± 1.05 Bc
CV (%)	6.07			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	***			
	Seed length (mm)			
US-802	13.9 ± 0.52 Ba	15.1 ± 0.29 Aa	14.5 ± 0.62 ABa	14.3 ± 0.53 Ba
US-897	8.7 ± 0.26 Bc	10.3 ± 0.16 Ac	10.2 ± 0.19 Ac	9.9 ± 0.18 Ac
US-942	10.5 ± 0.33 Bb	11.2 ± 0.31 ABb	11.3 ± 0.48 ABb	11.5 ± 0.20 Ab
CV (%)	3.19			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	**			
	Seed width (mm)			
US-802	7.0 ± 0.15 Ca	7.4 ± 0.29 Ba	7.5 ± 0.18 ABa	7.8 ± 0.28 Aa
US-897	5.2 ± 0.15 Cb	5.7 ± 0.08 Abb	5.8 ± 0.19 Ac	5.4 ± 0.16 BCc
US-942	5.9 ± 0.33 ABc	5.8 ± 0.11 Bb	6.2 ± 0.06 ABb	6.3 ± 0.12 Ab
CV (%)	3.13			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	***			
	Seed weight (mg)			
US-802	193.0 ± 4.35 Ca	206.7 ± 5.85 Ba	215.1 ± 5.25 Aa	173.3 ± 4.05 Da
US-897	62.9 ± 3.35 Cc	107.3 ± 2.78 Ac	105.7 ± 4.71 Ac	94.1 ± 4.27 Bc
US-942	99.5 ± 4.62 Cb	136.5 ± 3.64 ABb	137.1 ± 2.53 Ab	131.2 ± 4.55 Bb
CV (%)	3.08			
Rootstocks	***			
Harvest	***			
Rootstock × Harvest	***			

*Means followed by the same capital and lowercase letter in the row and column, respectively, do not significantly differ according to the Tukey's test ($P \leq 0.05$). Significant level: **$P \leq 0.01$; ***$P \leq 0.001$; ns, non-significant.*

mature (**Table 2**). The characteristics of the fruits may vary with the cropping season, due to biotic and abiotic factors, such as HLB (Rosales and Burns, 2011) or water stress (Garcia-Tejero et al., 2011), and may also depend on the fruit loading (Iglesias et al., 2007; Agustí and Primo-Millo, 2020). Fruits from US-802 were larger than those from US-897 and US-942, and had the highest number of seeds per fruit (33–50), depending on the season (**Table 3**). Number of seeds was different depending on the rootstock variety and fruit size, as previously reported (Alferez and Bordas, 2019). The number of seeds per fruit is one of the major factors for citrus rootstock selection. Citrus rootstocks that produce large number of seeds per fruit and good seedling emergence performance are preferred by the nurseries (Bisi et al., 2020); however, it is worth noting that US-942, one of the rootstocks currently most favored by citrus growers in Florida due to its superior performance under HLB conditions, produces significantly less seeds (**Table 3**). This adds an additional challenge to the citrus industry.

Seed size, including length and width, was also significantly ($P \leq 0.05$) variable during fruit maturation. In July, seeds were small for all evaluated rootstocks (**Table 3**). At that time, a considerable number of seeds had their outer coat (testa) still under the growing stage, thus being more sensitive to damage during storage (Carvalho and Silva, 2013). The seed coat is an important component of the seed, preventing dehydration and premature germination, as it offers resistance to the root apex emergence in the area of the micropyle (Agustí and Primo-Millo, 2020) and also allows seeds to last longer under appropriate storage conditions. The testa appeared totally formed in August for all three varieties, indicating increased seed viability with fruit maturation. Seed weight also fluctuated across the evaluated

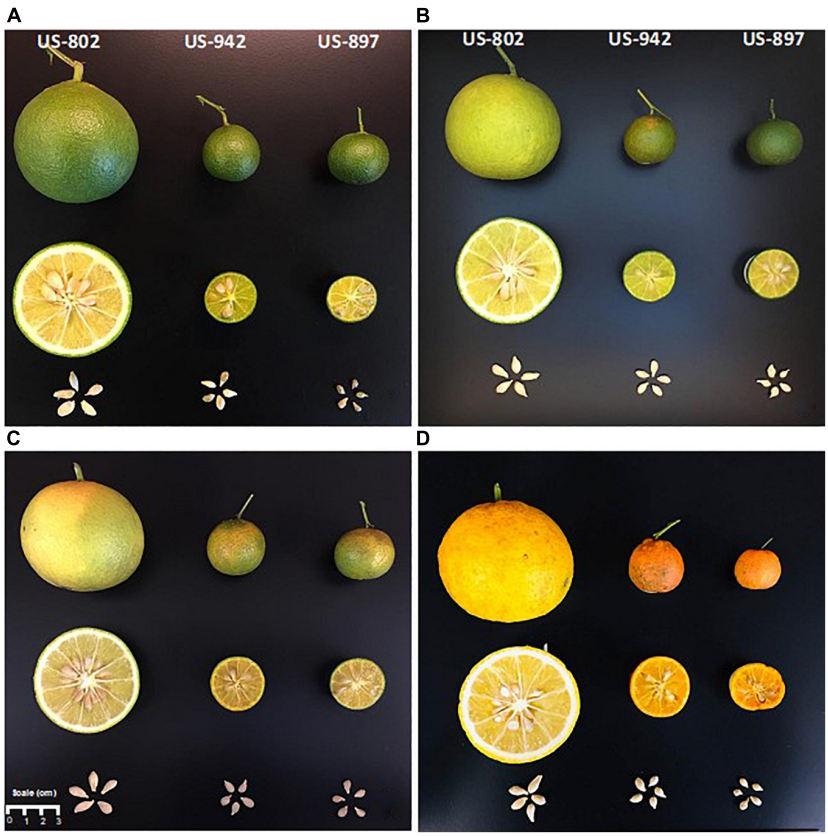

FIGURE 2 | Flavedo color development of citrus rootstock fruits and seed comparison during different harvest periods in mid-July **(A)**, mid-August **(B)**, mid-September **(C)** 2020, and mid-December **(D)** 2019.

period reaching the highest weights in August and September for all tested rootstocks. Seed quality parameters, such as size and weight, play a major role in germination and seedling establishment, and are positively correlated with seed vigor, as variability in seed size may contribute to the variance in seed germination performance (Guo et al., 2015).

Fruit Color Development

Color is one of the most important parameters to determine maturation of citrus fruit. However, color development in citrus rootstock fruit peel has not gained enough attention, since fruit is not marketable. In our study, we monitored the color change of the peel with the aim of determining if this parameter can be a good non-destructive indicator of seed maturity and hence, viability. Color change in flavedo was monitored during the entire fruit maturation period in all three varieties (**Figure 2**). The color development was quantitatively described based on lightness (L^*) and CCI attributes. The loss of the dark green color and the development of orange color was evident throughout the maturation period (**Figures 2**, **3**). An increase in L^* was observed in the flavedo as the fruit collection advanced through the season (**Figure 3A**). This increase was likely related to the chlorophyll degradation as described by Zhou et al. (2010). Fruit from US-802 had the highest L^* value, indicating both a lighter color intensity and earlier peel maturation as compared to fruits from US-897 and US-942. Color break in US-802 and US-942 was observed in August (**Figure 2B**), a month prior to US-897. By December, the L^* decreased only in the US-802 fruits; this might be attributed to the fact that, fruits started to lose water leading to a pressure loss over the peel, which may have affected flavedo lightness. In all varieties, CCI increased during the season. In the case of US-897, color change was delayed as compared to the other two rootstocks and was not evident until September (**Figure 2C**).

Seed Germination and Seedling Emergence

In seed lots with high viability, the ability to produce usable seedlings under less-than-optimal germination conditions is related to seed vigor. Seed lots can be classified as low or high vigor, depending on the degree of germination and emergence performance, particularly under unfavorable conditions (Guo et al., 2015). High vigor seeds are proxy of crop establishment and sustainable productivity (Araújo et al., 2016). Thus, seed vigor testing is an essential tool used to evaluate commercial seed lots. The most common vigor tests are based on germination behavior (Hampton and TeKrony, 1995). These include normal germination percentage after a stress imposition, germination speed (time to radicle protrusion), and early seedling growth

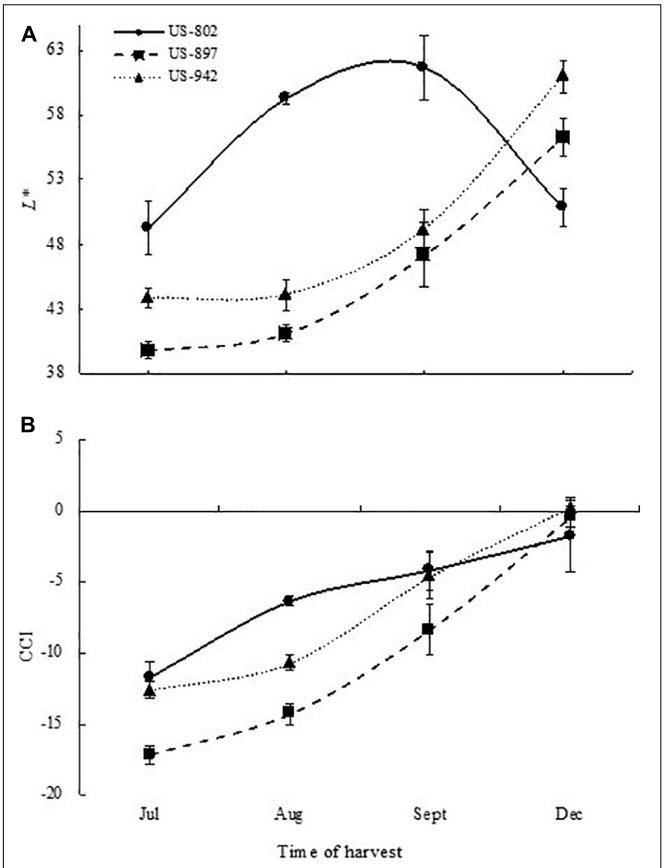

FIGURE 3 | Flavedo color development of citrus rootstock fruits during different harvest periods: Lightness (L^*) **(A)** and citrus color index (CCI) **(B)**.

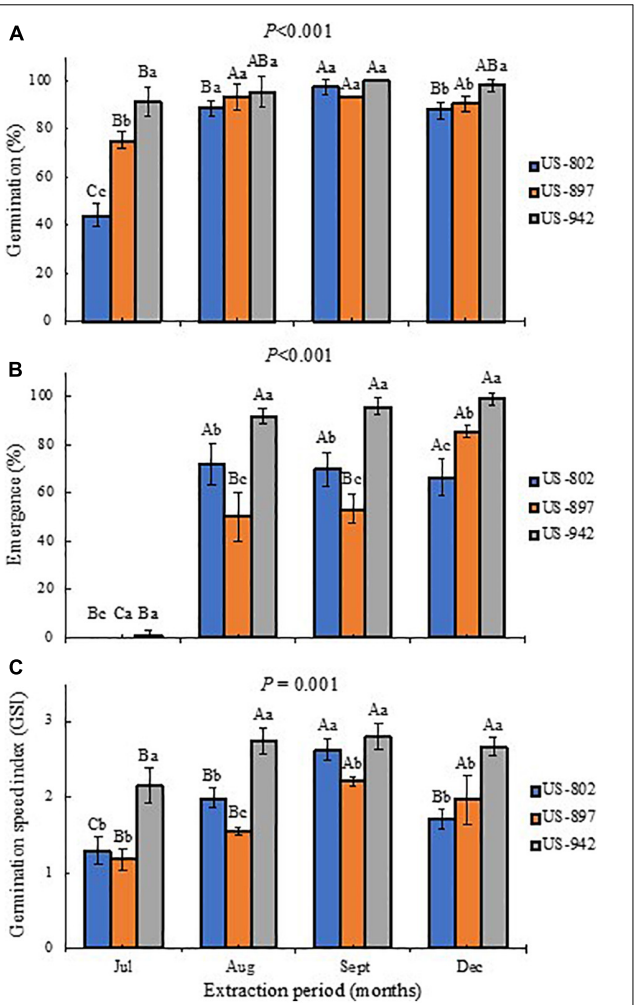

FIGURE 4 | Percentage of germination **(A)**, emergence **(B)**, and germination speed index **(C)** of three citrus rootstocks during different period of seed extraction at the Southwest Florida Research and Education Center, Immokalee, FL, United States. Bars followed by the same lowercase and capital letter do not significantly differ in regard to rootstock and seed extraction period, respectively, according to the Tukey's test ($P \leq 0.05$).

following germination. Germination speed has been used as an indicator of seed vigor, especially in seed priming experiments (Geneve, 2005), and it is an important measurement used to model seed germination (Bradford, 1990).

The stage of fruit maturation significantly affected the germination performance of the citrus rootstock seeds, irrespective of the variety (**Figure 4**). There was a significant interaction ($P \leq 0.001$) between rootstock and time of fruit harvest for almost all physiological characteristics evaluated. Seeds extracted in July had the lowest percentage of germination for all three rootstocks, particularly for the US-802, with less than 50% of the seeds able to germinate (**Figure 4A**). However, an increase in seed germination was observed when fruit harvest progressed (**Figure 4A**). Rootstock fruits harvested in August and September had seeds with higher germination potential, as more than 90% of the seeds generated seedlings (**Figure 4A**), which can be related to the seed quality parameters (size and weight), as larger seeds usually result in improved stand establishment and faster germination (Guo et al., 2015). This rate of germination was similar or even higher than that from the seeds obtained from fully mature fruits, harvested in December.

This germination trend was also observed for seedling emergence, under nursery standard conditions (**Figure 4B**). In July, the rootstock seedlings did not emerge, indicating that the seeds were not physiologically ready yet to be extracted from the fruits. Later, the seeds performed better, showing good seedling emergence progress, mainly for the US-942 rootstock. In general, seeds from fruits harvested in September showed the highest vigor according to the GSI, and root and SGRs (**Figures 4B**, **5B** and **Table 4**). All these parameters are important for rootstock seedlings production, as the time-consuming period required in this process is highly dependent on the seed vigor (Bowman et al., 2016a; Vashisth et al., 2020). Based on this, seeds all rootstocks were ready in August, showing US-942 more % of emergence than US-802 and US-897 (**Figure 4B**), resulting in faster seed germination (**Figure 4C**); In contrast, US-802 had the faster SGR (**Figure 5B**). Seeds from US-942 took about 24 days to reach 50% of the total seedling emergence (**Table 4**). In contrast, seed emergence for the US-897 and US-802 took 37 and 43 days, respectively (**Table 4**).

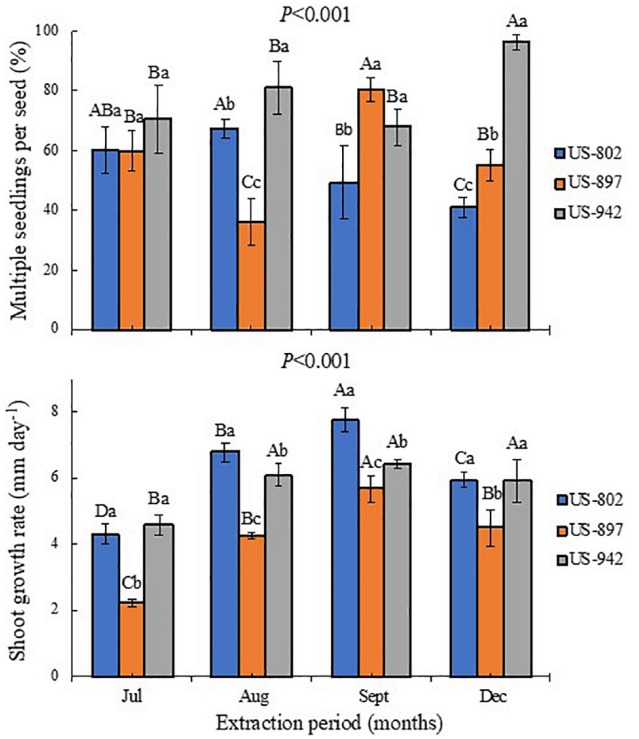

FIGURE 5 | Percentage of multiple seedlings per seed and shoot growth rate of three citrus rootstocks during different period of seed extraction at the Southwest Florida Research and Education Center, Immokalee, FL, United States. Bars followed by the same lowercase and capital letter do not significantly differ in regard to rootstock and seed extraction period, respectively, according to the Tukey's test ($P \leq 0.05$).

The seeds of the US-942 rootstock were also more efficient in generating multiple seedlings per seed (**Figure 5A**), an important aspect to be considered during rootstock production (Bisi et al., 2020). This effect increased as the fruit matured, as compared with the other two rootstocks. In addition to the zygotic embryo, the citrus seeds have nucellar embryos which are developed from somatic nucellus cells (Frost et al., 1968). Polyembryonic seeds usually contain between 2 and 10 nucellar embryos, though, sometimes, they may contain more embryos (Agustí and Primo-Millo, 2020). This characteristic allows to propagate uniform and clonal (true-to-type) rootstock seedlings, as nucellar seedlings are genetically identical to the maternal parent (Kepiro and Roose, 2010). The seed capacity to produce one or more embryos depends on its physiological quality, which is related to the fruit maturation, and nutritional and healthiness conditions of the trees and seeds (Carvalho and Silva, 2013).

Correlation Analysis

In order to compare such divergent rootstocks, a correlation matrix was built for each single citrus rootstock (US-802, US-897, and US-942) based on Pearson's correlation coefficients (r) and the studied traits (**Figures 6–8** and **Supplementary Figure 1**). Significant positive correlations ($P \leq 0.05$) were found (**Figure 6** and **Supplementary Figure 1**) between RGR and seed weight ($r = 0.98$), and number of seeds ($r = 0.98$) for the US-802, but strongly negative with fruit shape ($r = -0.96$). However, no more obvious correlations were observed between all other traits for this citrus rootstock. The correlation analysis for US-897 revealed a strong positive correlation between germination and seed weight ($r = 0.99$, $P \leq 0.05$), and seed length ($r = 1.00$; $P \leq 0.01$) (**Figure 7**), as well as between days to reach 50% of the total emergence (T50) and seed length ($r = 0.95$; $P \leq 0.05$). No significant negative correlations ($P \leq 0.05$) were found between the evaluated traits for the US-897. Finally, for US-942 numerous positive and negative correlations were observed among its dimensions (**Figure 8**). Seed weight was positively ($P \leq 0.05$) correlated with GSI ($r = 0.99$), SGR ($r = 0.99$), and emergence ($r = 0.98$). Similarly, the CCI was highly and positively correlated with fruit weight ($r = 0.99$; $P \leq 0.01$), fruit diameter ($r = 0.98$; $P \leq 0.05$), fruit length ($r = 0.95$; $P \leq 0.05$), and seed width ($r = 0.98$; $P \leq 0.05$). Furthermore, the US-942 fruit weight and size (length and diameter) were positively ($P \leq 0.05$) correlated with seed length ($r \geq 0.97$) and width ($r \geq 0.95$) but negatively associated with the number of seeds ($r \leq -0.93$). Negative correlation was also found for fruit shape and polyembryony ($r = -0.99$; $P \leq 0.05$).

In general, seed weight was highly correlated with the germination and emergence dimensions for all three evaluated citrus rootstocks. Previous studies have showed strong relationship between seed weight and seedling growth measurements, including germination and emergence, among different species (Hanley et al., 2007; Huang et al., 2017; Poletto et al., 2018). The control of seed mass and size are regulated by genetic and epigenetic pathways, but it has been little investigated in woody perennial plants (Poletto et al., 2018).

TABLE 4 | Seedling root growth rate and days to reach 50% of the total emergence of three citrus rootstocks during different period of seed extraction at the Southwest Florida Research and Education Center, Immokalee, FL, United States (mean value ± standard deviation).

Source of variance	Root growth rate (mm day^{-1})	T50 (days)[1]
Rootstock		
US-802	3.87 ± 0.51 B[2]	43 ± 4.96 A
US-897	3.04 ± 0.54 C	37 ± 5.37 B
US-942	4.61 ± 0.47 A	24 ± 6.43 C
Harvest		
July	3.87 ± 0.60 b	38 ± 0.01 a
August	3.74 ± 0.77 b	31 ± 10.57 a
September	4.42 ± 0.67 a	31 ± 8.77 a
December	3.32 ± 0.79 c	39 ± 7.68 a
CV (%)	8.32	24.15
Rootstock	***	***
Harvest	***	***
Rootstock × harvest	ns	ns

[1] *T50, days to reach 50% of the total emergence.*
[2] *Means followed by the same letter in the column do not significantly differ according to the Tukey's test ($P \leq 0.05$).*
*Significant level: ***$P \leq 0.001$; ns, non-significant.*

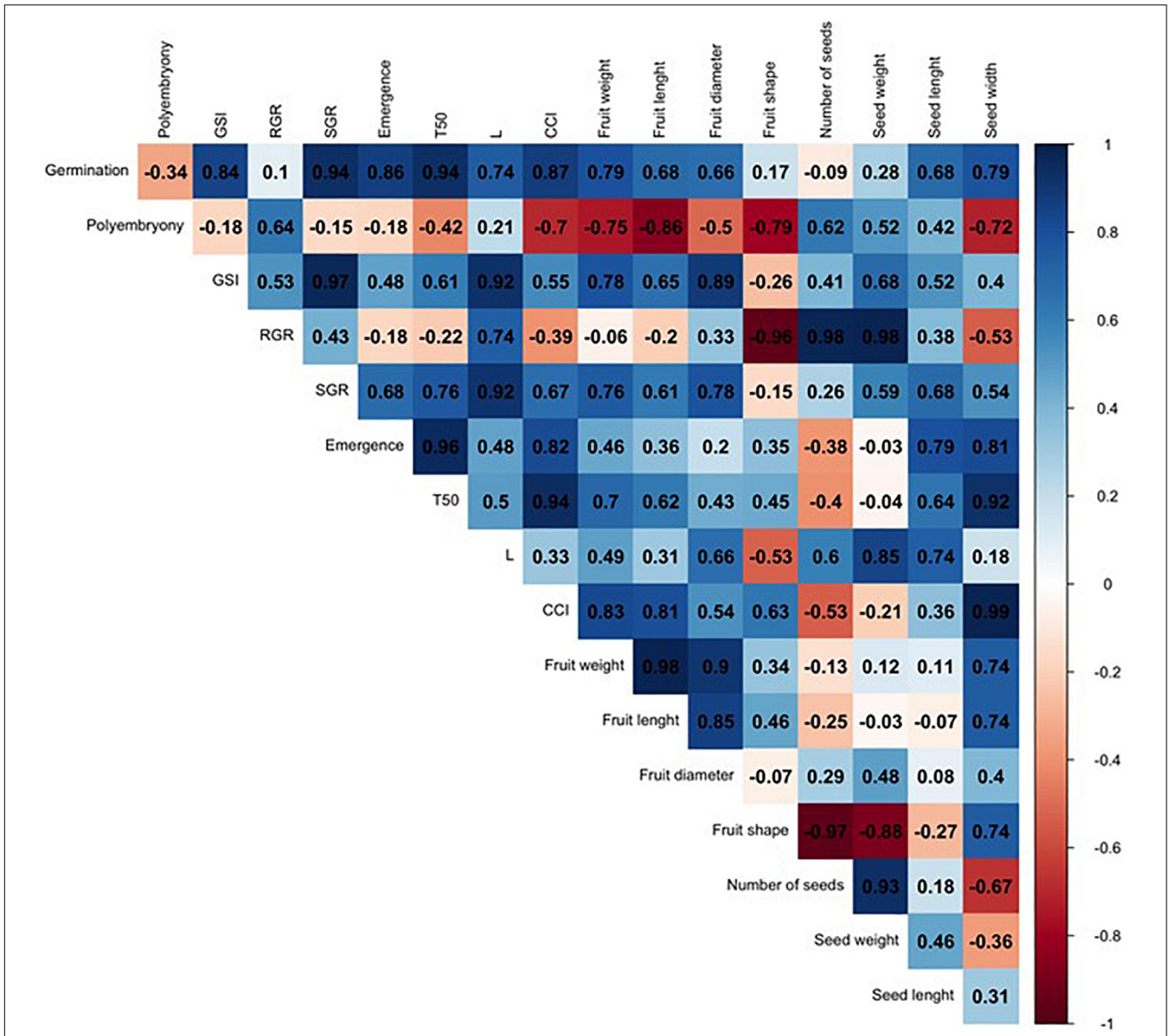

FIGURE 6 | Correlation analysis among the variables studied for US-802 rootstock. The numbers display the Pearson's correlation coefficient (r). Blue cells indicate positive correlations among the variables (1) and the red cells indicate negative correlations (–1). The color density and numbers reflect the scale of correlation. Variables: germination; polyembryony; GSI: germination speed index; RGR: root growth rate; SGR: shoot growth rate; emergence; T50: days to reach 50% of the total emergence; *L*: lightness value; CCI: citrus color index; fruit weight; fruit length; fruit diameter; fruit shape; number of seeds per fruit, seed weight; seed length; and seed width.

Current evidence has shown that the homolog of *Auxin Response Factor 19* (*JcARF19*) significantly increases seed size and mass in *Jatropha curcas* plants, confirming the importance of the auxin pathway in controlling these seed attributes in woody perennial plants (Sun et al., 2017). Hanley et al. (2007) reinforce that larger seeds are usually assumed to be more advantageous for seedling establishment than smaller-seeded species, as they are less dependent on soil nutrient availability at the initial growth stage. Indeed, this relationship appears to be triggered by the seed vigor, an important index of seed quality, as larger and heavier seed usually generates more vigorous seedlings (Poletto et al., 2018; Wen et al., 2018). Moreover, this index measures the potential for a rapid and uniform seedling emergence and may reduce seed production risks associated with poor stand establishment (Marcos-Filho, 2015; Wen et al., 2018), resulting in a better management decision.

US-942 seed weight has shown strong relationships with most germination and emergence assessments (**Figure 8**) evidencing its vigorous potential as have been reported previously (Bowman and Joubert, 2020). Furthermore, this

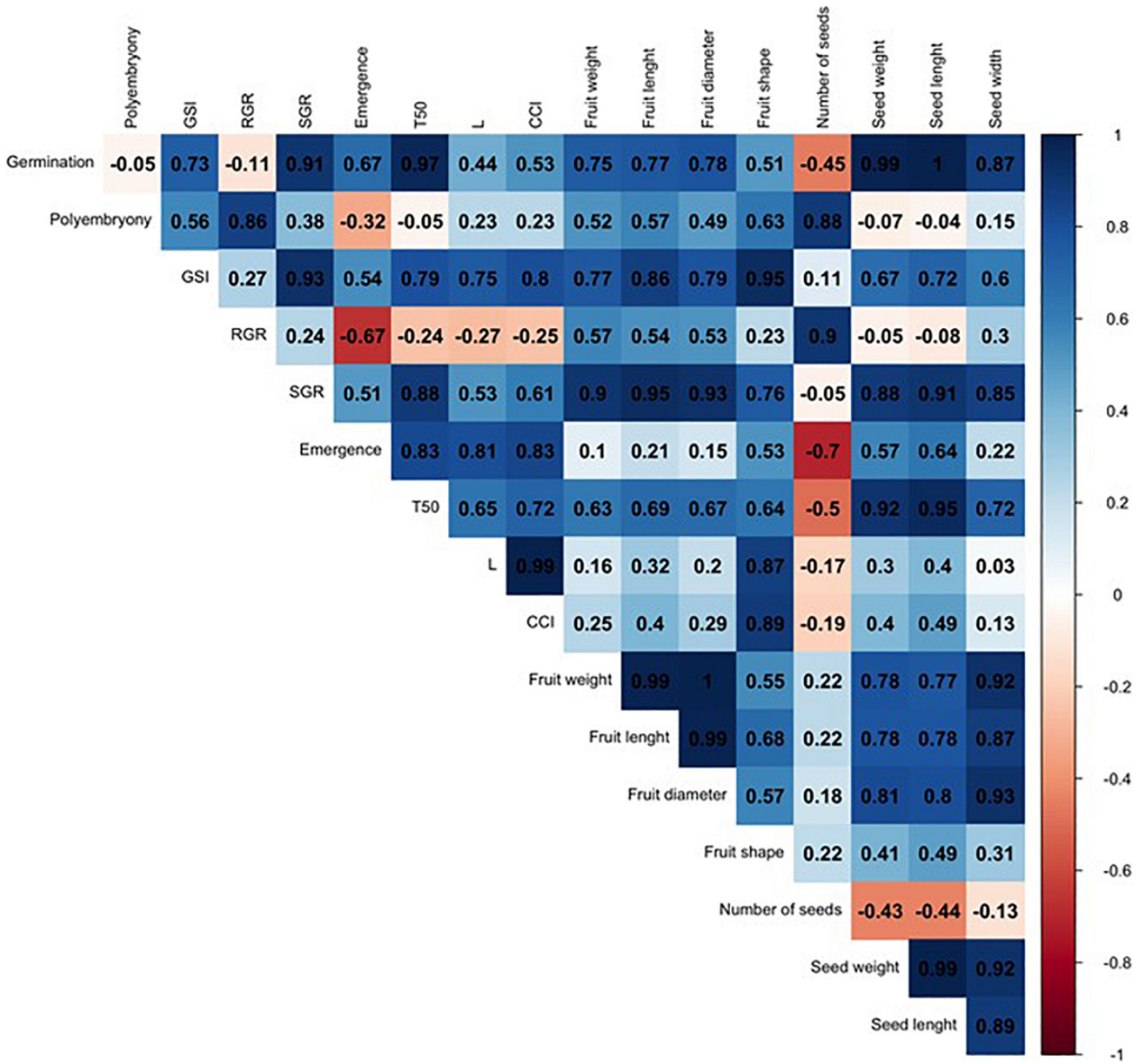

FIGURE 7 | Correlation analysis among the variables studied for US-897 rootstock. The numbers display the Pearson's correlation coefficient (r). Blue cells indicate positive correlations among the variables (1) and the red cells indicate negative correlations (–1). The color density and numbers reflect the scale of correlation. Variables: germination; polyembryony; GSI: germination speed index; RGR: root growth rate; SGR: shoot growth rate; emergence; T50: days to reach 50% of the total emergence; *L*: lightness value; CCI: citrus color index; fruit weight; fruit length; fruit diameter; fruit shape; number of seeds per fruit, seed weight; seed length; and seed width.

rootstock provided superior field performance to various other high-demanded citrus rootstocks under Florida soil-climate condition and HLB-pressure (Bowman et al., 2016a,b).

Assessing Abscission Capacity

Color data, when taken together and compared with seed emergence and germination data suggest that color change in the peel is not the best indicator of seed maturity for these rootstock varieties, because on one hand, fruit from US-802 changed color 1 month earlier than fruit from US-897, but seed germination was not different by August, and on the other hand, fruit from US-942 and US-802 changed color at the same time, but GSI in US-942 were significantly higher than in US-802. With maturation, fruit enters senescence and ultimately abscission, two processes that are genetically determined and hormonally regulated. Citrus fruits, although non-climacteric, can respond to ethylene, and this response is highly coordinated and may involve abscission (Alferez and Zacarias, 1999; Alférez et al., 2006; Alferez et al., 2021b); in this sense, increased capacity for abscission could indicate

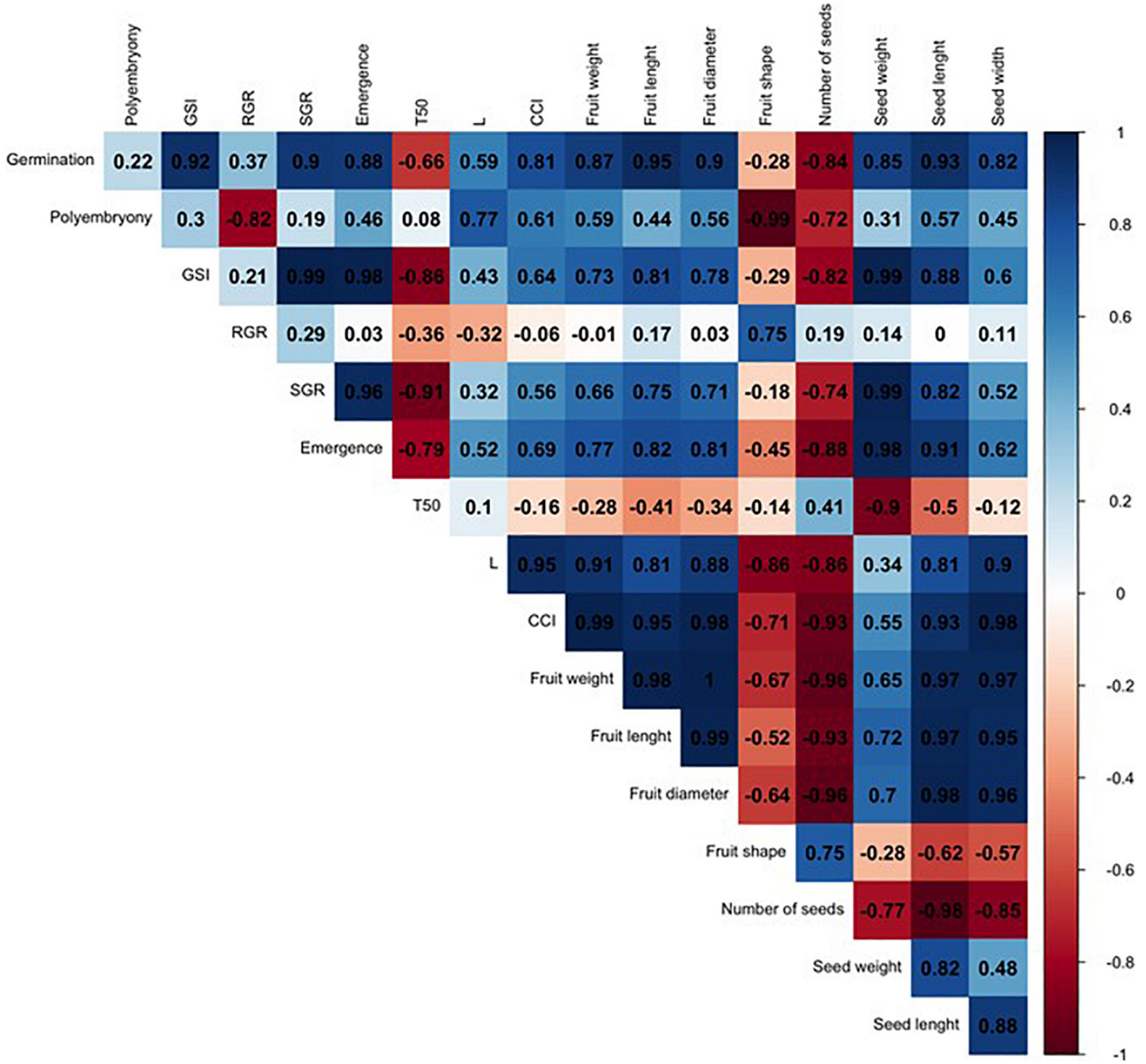

FIGURE 8 | Correlation analysis among the variables studied for US-942 rootstock. The numbers display the Pearson's correlation coefficient (r). Blue cells indicate positive correlations among the variables (1) and the red cells indicate negative correlations (–1). The color density and numbers reflect the scale of correlation. Variables: germination; polyembryony; GSI: germination speed index; RGR: root growth rate; SGR: shoot growth rate; emergence; T50: days to reach 50% of the total emergence; *L*: lightness value; CCI: citrus color index; fruit weight; fruit length; fruit diameter; fruit shape; number of seeds per fruit, seed weight; seed length; and seed width.

when the seed is ready to germinate, as fruit abscission is a mechanism for seed dispersal in nature. To further confirm this idea, we studied *in vitro* the fruit abscission response during maturation in the three rootstocks. We compared the abscission response of fruit from the three rootstocks to the ethylene precursor ACC during maturation in time course experiments monitoring abscission rate. We found that all three rootstocks started to respond to ACC in advancing abscission as early as July, but response was maximized in August; Fruit from US-942 incubated with water (**Figure 9** upper central panel) reached 100% abscission by day 6 of incubation, whereas after ACC treatment this time was reduced to 4 days; in the case of US-897, the abscission response after ACC treatment was advanced from 9 to 4 days in fruit harvested in August, whereas in fruit from US-802 abscission was advanced from 10 to 7 days, showing a milder response to the ethylene precursor This set of data further supports the notion that seeds from all three varieties may be ready to be harvested as early as August (**Figure 9**).

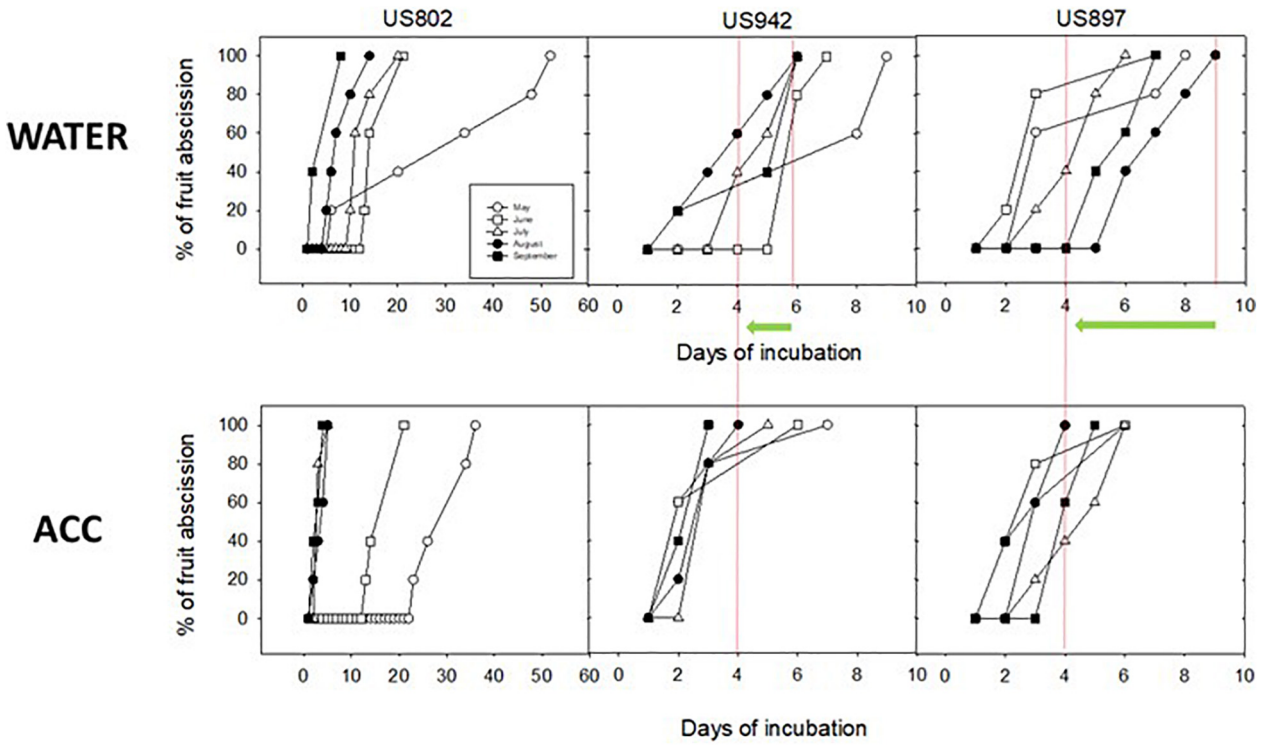

FIGURE 9 | Abscission dynamics of fruit from US-802, US-897, and US-942 harvested on May, June, July, August, and September, and incubated *in vitro* in the absence (lower panels, water control) or presence (upper panels) of the ethylene precursor ACC (1-aminocyclopropane-l-carboxylic acid). The 3 panels are aligned to allow comparisons of abscission rate. Green arrows and red lines highlight the advancement in fruit abscission rate for August.

CONCLUSION

The physiological response on rootstock seed germination is affected by the maturation of the fruits at harvest. For Florida conditions, fruits from all three varieties can be harvested as early as August without losing any germination potential based on seed vigor parameters studied here and on our study of abscission dynamics. Therefore, fruits from these rootstock varieties can be harvested for seed extraction before the peak of the hurricane season, avoiding fruit drop and eliminating the risk of not having enough seeds for nursery supply. Together, this constitutes a new managerial tool for nurseries in Florida to control and adjust operations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FA conceived the project and got the funding. DC, DB, and TG performed the research. DC, FA, and RL analyzed the data. DC and FA wrote the manuscript. All authors accepted the final manuscript.

FUNDING

This work was partially funded by a grant from the Florida Nursery, Growers and Landscape Association (FNGLA). Support for DC at Alferez's lab in SWFREC, UF/IFAS was provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) [Grant No. 88881.361958/2019-01].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.777078/full#supplementary-material

Supplementary Figure 1 | Scatterplot and correlation matrix of the studied variables for US-802, US-897, and US-942 rootstocks. The matrix contains of pairwise scatterplots for the following variables: GERM: germination; POLY: polyembryony; GSI: germination speed index; RGR: root growth rate; SGR: shoot growth rate; EMER: emergence; T50: days to reach 50% of the total emergence; L: lightness value; CCI: citrus color index; FW: fruit weight; FL: fruit length; FD: fruit diameter; FS: fruit shape; NS: number of seeds per fruit; SWe: seed weight; SL: seed length; and SWi: seed width. Significant level: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

REFERENCES

Agustí, M., and Primo-Millo, E. (2020). "Flowering and fruit set," in *The Genus Citrus*, eds M. Talon, M. Caruso, and F. G. Jr. Gmitter (Cambridge: Woodhead Publishing), 219–244. doi: 10.1016/B978-0-12-812163-4.00011-5

Albrecht, U., Bodaghi, S., Meyering, B., and Bowman, K. D. (2020). Influence of rootstock propagation method on traits of grafted sweet orange trees. *HortScience* 55, 729–737. doi: 10.21273/HORTSCI14928-20

Albrigo, L. G., Buker, R. S., Burns, J. K., Castle, W. S., Futch, S., Mccoy, C. W., et al. (2005). The impact of four hurricanes in 2004 on the Florida citrus industry: experiences and lessons learned. *Proc. Fla. State Hort. Soc.* 118, 66–74.

Alferez, F. (2018). Citrus resilience under a CAT 4 hurricane: lessons from Irma in South West Florida. *Hortscience* 53:S201.

Alferez, F., and Bordas, M. (2019). Citrus nursery production guide, chapter 7: seed and budwood production, transport, and conservation. *EDIS* 2019:3. doi: 10.32473/edis-hs1336-2019

Alferez, F., and Zacarias, L. (1999). "Interaction between ethylene and abscisic acid in the regulation of citrus fruit maturation," in *Biology and Biotechnology of the Plant Hormone Ethylene II*, eds A. K. Kanellis, C. Chang, H. Klee, A. B. Bleecker, J. C. Pech, and D. Grierson (Amsterdam: Kluwer Academic Publishers), 183–184. doi: 10.1007/978-94-011-4453-7_30

Alferez, F., Carvalho, D. U., and Boakye, D. A. (2021b). Interplay between abscisic acid and gibberellins, as related to ethylene and sugars, in regulating maturation of non-climacteric fruit. *Int. J. Mol. Sci.* 22:669. doi: 10.3390/ijms22020669

Alferez, F., Carvalho, D. U., Boakye, D. A., Gast, T., and Dutt, M. (2021a). Maximizing rootstock seed production. *Citrus Ind.* 102, 22–25.

Alférez, F., Pozo, L., and Burns, J. K. (2006). Physiological changes associated with senescence and abscission in mature citrus fruit induced by 5-chloro-3-methyl-4-nitro-1H-pyrazole and ethephon application. *Physiol. Plant.* 127, 66–73. doi: 10.1111/j.1399-3054.2006.00642.x

Araújo, S. D. S., Paparella, S., Dondi, D., Bentivoglio, A., Carbonera, D., and Balestrazzi, A. (2016). Physical methods for seed invigoration: advantages and challenges in seed technology. *Front. Plant Sci.* 7:646. doi: 10.3389/fpls.2016.00646

Bagio, T. Z., Canteri, M. G., and Leite Júnior, R. P. (2020). Revisiting seed transmission of '*Candidatus* Liberibacter asiaticus' in different citrus species and hybrids. *Trop. Plant Pathol.* 45, 334–341. doi: 10.1007/s40858-020-00348-7

Belasque Júnior, J., Bergamin Filho, A., Bassanezi, R. B., Barbosa, J. C., Fernandes, N. G., Yamamoto, P. T., et al. (2009). Base científica para a erradicação de plantas sintomáticas e assintomáticas de Huanglongbing (HLB, Greening) visando o controle efetivo da doença. *Trop. Plant Pathol.* 34, 137–145. doi: 10.1590/S1982-56762009000300001

Bisi, R. B., Albrecht, U., and Bowman, K. D. (2020). Seed and seedling nursery characteristics for 10 USDA citrus rootstocks. *HortScience* 55, 528–532. doi: 10.21273/HORTSCI14598-19

Bowman, K. D., and Joubert, J. (2020). "Citrus rootstocks," in *The Genus Citrus*, eds M. Talon, M. Caruso, and F. G. Jr. Gmitter (Cambridge: Woodhead Publishing), 105–127. doi: 10.1016/B978-0-12-812163-4.00006-1

Bowman, K. D., Faulkner, L., and Kesinger, M. (2016a). New citrus rootstocks released by USDA 2001–2010: field performance and nursery characteristics. *HortScience* 51, 1208–1214. doi: 10.21273/HORTSCI10970-16

Bowman, K. D., McCollum, G., and Albrecht, U. (2016b). Performance of 'Valencia' orange (*Citrus sinensis* [L.] Osbeck) on 17 rootstocks in a trial severely affected by huanglongbing. *Sci. Hortic.* 201, 355–361. doi: 10.1016/j.scienta.2016.01.019

Bradford, K. J. (1990). A water relations analysis of seed germination rates. *Plant Physiol.* 94, 840–849. doi: 10.1104/pp.94.2.840

Brown, R. F., and Mayer, D. G. (1986). A critical analysis of Maguire's germination rate index. *J. Seed Technol.* 10, 101–110.

Carvalho, S. A., and Silva, L. F. C. (2013). Monitoring the viability of citrus rootstocks seeds stored under refrigeration. *Rev. Bras. Frutic.* 35, 238–245. doi: 10.1590/S0100-29452013000100027

Chaires, P. (2017). Citrus nurseries analyze the need for seed. *Growing Produce*, February 2017.

Etxeberria, E., Gonzalez, P., Achor, D., and Albrigo, G. (2009). Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees. *Physiol. Mol. Plant Pathol.* 74, 76–83. doi: 10.1016/j.pmpp.2009.09.004

Frost, H. B., Soost, R. K., Reuther, W., Batchelor, L. D., and Webber, H. J. (1968). *The Citrus Industry*. Berkeley, CA: University of California.

Garcia-Tejero, I., Duran-zuazo, V. H., Arriaga-Sevilla, J., and Muriel-Fernandez, J. L. (2011). Impact of water stress on citrus yield. *Agron. Sustain. Dev.* 32, 651–659. doi: 10.1007/s13593-011-0060-y

Geneve, R. L. (2005). "Vigor testing in flower seeds," in *Flower Seeds, Biology and Technology*, eds M. B. McDonald and F. Kwong (London: CAB International), 311–332. doi: 10.1079/9780851999067.0311

Graham, J. H. G., Johnson, E. G., Gottwald, T. R., and Irey, M. S. (2013). Presymptomatic fibrous root decline in citrus trees caused by huanglongbing and potential interaction with *Phytophthora* spp. *Plant Dis.* 97, 1195–1199. doi: 10.1094/PDIS-01-13-0024-RE

Guo, J., Suo, S., and Wang, B. S. (2015). Sodium chloride improves seed vigour of the euhalophyte *Suaeda salsa*. *Seed Sci. Res.* 25, 335–344. doi: 10.1017/S0960258515000239

Hampton, J. G., and TeKrony, D. M. (1995). *Handbook of Vigour Test Methods*. Zurich: International Seed Testing Association.

Hanley, M. E., Cordier, P. K., May, O., and Kelly, C. K. (2007). Seed size and seedling growth: differential response of Australian and British Fabaceae to nutrient limitation. *New Phytol.* 174, 381–388. doi: 10.1111/j.1469-8137.2007.02003.x

Huang, M., Zhang, R., Chen, J., Cao, F., Jiang, L., and Zou, Y. (2017). Morphological and physiological traits of seeds and seedlings in two rice cultivars with contrasting early vigor. *Plant Prod. Sci.* 20, 95–101. doi: 10.1080/1343943X.2016.1229571

Husson, F., Lê, S., and Pages, J. (2017). *Exploratory Multivariate Analysis by Example Using R*. Boca Raton, FL: Chapman and Hall. doi: 10.1201/b21874

Iglesias, D. J., Cercós, M., Colmenero-Flores, J. M., Naranjo, M. A., Ríos, G., Carrera, E., et al. (2007). Physiology of citrus fruiting. *Braz. J. Plant Physiol.* 19, 333–362. doi: 10.1590/S1677-04202007000400006

Jiménez-Cuesta, M., Cuquerella, J., and Martínez-Jávega, J. M. (1981). Determination of a color index for citrus fruit degreening. *Proc. Int. Soc. Citriculture* 2, 750–753.

Kepiro, J. L., and Roose, M. L. (2010). AFLP markers closely linked to a major gene essential for nucellar embryony (apomixis) in *Citrus maxima* × *Poncirus trifoliata*. *Tree Genet. Genomes* 6, 1–11. doi: 10.1007/s11295-009-0223-z

Lê, S., Josse, J., and Husson, F. (2008). FactoMineR: an R package for multivariate analysis. *J. Stat. Softw.* 25, 1–18. doi: 10.18637/jss.v025.i01

Maguire, J. D. (1962). Speed of germination—aid in selection and evaluation for seedling emergence and vigor 1. *Crop Sci.* 2, 176–177. doi: 10.2135/cropsci1962.0011183X000200020033x

Marcos-Filho, J. (2015). Seed vigor testing: an overview of the past, present and future perspective. *Sci. Agric.* 72, 363–374. doi: 10.1590/0103-9016-2015-0007

McGuire, R. G. (1992). Reporting of objective color measurements. *HortScience* 27, 1254–1255. doi: 10.21273/HORTSCI.27.12.1254

Merelo, P., Agustí, J., Arbona, V., Costa, M. L., Estornell, L. H., Gómez-Cadenas, A., et al. (2017). Cell wall remodeling in abscission zone cells during ethylene-promoted fruit abscission in citrus. *Front. Plant Sci.* 8:126. doi: 10.3389/fpls.2017.00126

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Orbovic, V., Dutt, M., and Grosser, J. W. (2011). Seasonal effects of fruit age on regeneration potential and transformation success rate in three citrus cultivars. *Sci. Hortic.* 127, 262–266. doi: 10.1016/j.scienta.2010.10.004

Orbovic, V., Dutt, M., and Grosser, J. W. (2013). Evaluation of the germination potential of citrus seeds during the harvesting season. *HortScience* 48, 1197–1199. doi: 10.21273/HORTSCI.48.9.1197

Pokhrel, S., Meyering, B., Bowman, K. D., and Albrecht, U. (2021). Horticultural attributes and root architectures of field-grown 'Valencia' trees grafted on different rootstocks propagated by seed, cuttings, and tissue culture. *HortScience* 56, 163–172. doi: 10.21273/HORTSCI15507-20

Poletto, T., Stefenon, V. M., Poletto, I., and Muniz, M. F. B. (2018). Pecan propagation: seed mass as a reliable tool for seed selection. *Horticulturae* 4:26. doi: 10.3390/horticulturae4030026

Pustika, A. B., Subandiyah, S., Holford, P., Beattie, G. A. C., Iwanami, T., and Masaoka, Y. (2008). Interactions between plant nutrition and symptom expression in mandarin trees infected with the disease huanglongbing. *Australas. Plant Dis. Notes* 3, 112–115. doi: 10.1071/DN08045

Rosales, R., and Burns, J. K. (2011). Phytohormone changes and carbohydrate status in sweet orange fruit from huanglongbing-infected trees. *J. Plant Growth Regul.* 30, 312–332. doi: 10.1007/s00344-011-9193-0

Sun, Y., Wang, C., Wang, N., Jiang, X., Mao, H., Zhu, C., et al. (2017). Manipulation of *Auxin Response Factor 19* affects seed size in the woody perennial *Jatropha curcas*. *Sci. Rep*. 7:40844. doi: 10.1038/srep40844

Vashisth, T., Chun, C., and Ozores-Hampton, M. (2020). Florida citrus nursery trends and strategies to enhance production of field-transplant ready citrus plants. *Horticulturae* 6:8. doi: 10.3390/horticulturae6010008

Wei, T., Simko, V., Levy, M., Xie, Y., Jin, Y., and Zemla, J. (2017). Package 'corrplot'. *Statistician* 56:e24.

Wen, D., Hou, H., Meng, A., Meng, J., Xie, L., and Zhang, C. (2018). Rapid evaluation of seed vigor by the absolute content of protein in seed within the same crop. *Sci. Rep*. 8:5569. doi: 10.1038/s41598-018-23909-y

Zhou, J. Y., Sun, C. D., Zhang, L. L., Dai, X., Xu, C. J., and Chen, K. S. (2010). Preferential accumulation of orange-colored carotenoids in Ponkan (*Citrus reticulata*) fruit peel following postharvest application of ethylene or ethephon. *Sci. Hortic.* 126, 229–235. doi: 10.1016/j.scienta.2010.07.019

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 December 2021
doi: 10.3389/fpls.2021.734430

Detection of Root Physiological Parameters and Potassium and Calcium Currents in the Rhizoplane of the Apple Rootstock Superior Line 12-2 With Improved Apple Replant Disease Resistance

*Yunfei Mao, Yijun Yin, Xueli Cui, Haiyan Wang, Xiafei Su, Xin Qin, Yangbo Liu, Yanli Hu and Xiang Shen**

National Key Laboratory of Crop Biology, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai'an, China

OPEN ACCESS

Edited by:
Vittorino Novello, University of Turin, Italy

Reviewed by:
Xiangming Xu, East Malling Research (EMR), United Kingdom
Shaikhul Islam, Ehime University, Japan
Salar Farhangi-Abriz, University of Tabriz, Iran

***Correspondence:**
Xiang Shen
shenx@sdau.edu.cn

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

Received: *15 July 2021*
Accepted: *29 November 2021*
Published: *17 December 2021*

Citation:
Mao Y, Yin Y, Cui X, Wang H, Su X, Qin X, Liu Y, Hu Y and Shen X (2021) Detection of Root Physiological Parameters and Potassium and Calcium Currents in the Rhizoplane of the Apple Rootstock Superior Line 12-2 With Improved Apple Replant Disease Resistance. Front. Plant Sci. 12:734430. doi: 10.3389/fpls.2021.734430

The cultivation of resistant rootstocks is one of the more effective ways to mitigate apple replant disease (ARD). We performed an ion current test, a pot experiment, and a pathogen infection test on the apple rootstocks 12-2 (self-named), T337, and M26. The ion current test showed that exposure to ARD soil extract for 30 min had a significant effect on K^+ ion currents at the meristem, elongation, and mature zones of the M26 rhizoplane and on Ca^{2+} currents in the meristem and elongation zones. ARD also had a significant effect on Ca^{2+} currents in the meristem, elongation, and mature zones of the T337 rhizoplane. Exposure to ARD soil extract for 5 min had a significant effect on K^+ currents in the meristem, elongation, and mature zones of 12-2 and on the Ca^{2+} currents in the elongation and mature zones. Compared to a 5-min exposure, a 30-min exposure to ARD extract had a less pronounced effect on K^+ and Ca^{2+} currents in the 12-2 rhizoplane. The pot experiment showed that ARD soil had no significant effect on any root architectural or physiological parameters of 12-2. By contrast, ARD soil significantly reduced some root growth indices and the dry and fresh weights of T337 and M26 compared with controls on sterilized soil. ARD also had a significant effect on root metabolic activity, root antioxidant enzyme activity (except superoxide dismutase for T337), and malondialdehyde content of T337 and M26. Pathogen infection tests showed that *Fusarium proliferatum* MR5 significantly affected the root structure and reduced the root metabolic activity of T337 and M26. It also reduced their root antioxidant enzyme activities (except catalase for T337) and significantly increased the root malondialdehyde content, reactive oxygen levels, and proline and soluble sugar contents. By contrast, MR5 had no such effects on 12-2. Based on these results, 12-2 has the potential to serve as an important ARD-resistant rootstock.

Keywords: cultivation of resistant rootstocks, ARD, potassium and calcium currents in rhizosphere, root physiological indices, pathogen infection

INTRODUCTION

Apple replant disease (ARD) is a common occurrence in major apple producing areas worldwide (Mazzola and Manici, 2012; Chen et al., 2020) and has significantly limited the sustainable development of apple production (Narwal, 2010; Mao et al., 2021b). Studies have shown that ARD is caused by a complex of soil microorganisms (Mazzola and Manici, 2012; Wang et al., 2021). The ARD pathogen complex consists of oomycetes, including *Pythium* and *Phytophthora*, and fungi such as *Ilyonectria* and *Rhizoctonia*, at times acting in concert with the lesion nematode *Pratylenchus penetrans* (Zhu et al., 2016). However, the specific pathogen complex may vary across geographic regions, or even between orchards in the same region (Zhou et al., 2021). Previous studies have shown that specialized *Fusarium* spp. are the pathogenic fungi that pose the greatest threat during continuous cropping of many plants (Duan et al., 2016; Xiang et al., 2021). *Fusarium* can cause necrosis and decay of plant roots, resulting in dwarfed plants, wilting, and even tree death (Wang G. S. et al., 2018; Xiang et al., 2021). Liu (2013) and Wang G. S. et al. (2018) have shown that *Fusarium* is one of the main causes of ARD in apple orchards in the Bohai Bay region of China. Zou et al. (2014) identified *Fusarium proliferatum* and other suspected pathogenic *Fusarium* spp. from apple orchard soils in Hebei Province, China. Recently, the specialized, ARD-associated *F. proliferatum* strain MR5 (MW600437.1) has been screened, identified, and shown to be highly pathogenic to apple roots (in review).

Although there are many ways to improve ARD symptoms (Pan et al., 2017), the development of improved ARD-resistant rootstocks is a long-term effective measure to prevent and control ARD (Bowen et al., 2010). The cultivation of resistant rootstocks can effectively control pests and diseases in the replanted soil, alleviate ARD caused by certain pathogenic bacteria (Rivard et al., 2012), strengthen plant stress resistance, and increase fruit yield and quality (Zhu and Saltzgiver, 2020). There have been many studies of resistant rootstocks in Europe, the United States, and other countries. Leinfelder and Merwin (2006) reported that the growth of G30 and CG6210 plants increased significantly and steadily, and the average lifespan of the CG6210 root system was five times greater than that of M7 after 4 years. Rumberger et al. (2004) reported similar results when grafting the Royal Empire variety onto three CG rootstocks (CG16, CG30, and CG210) and two M rootstocks (M7 and M26). Rootstocks G11, G16, and G41, which were developed within the Geneva rootstock-breeding program, are reportedly tolerant to some of the causative agents implicated in ARD, although this assessment has not been confirmed consistently in all studies (Reim et al., 2019). Nonetheless, for various reasons, these rootstocks have not been promoted in China. Instead, the dwarf rootstocks T337 and M26 are still the main apple rootstocks used for production in China. T337 offers the advantages of early fruiting and large yields (Fallahi et al., 2001), and M26 has higher graft compatibility and stronger healing ability (Wang et al., 2019a). However, both these rootstocks have short lifespans and shallow root systems, and they are generally considered to be ARD "susceptible" rootstocks (Wang et al., 2019a). Therefore, it is important to specifically select and use ARD resistant rootstocks in the main apple producing areas of China.

The results of our previous research on K^+ and Ca^{2+} absorption in the root meristem zones of three apple rootstock seedlings [*Malus hupehensis* Rehd., *Malus sieversii* (Ledeb.) Roem., and *Malus prunifolia* (Willd.) Borkh.] under replant stress showed that the greater the rootstock resistance, the less it was affected by ARD (Guo et al., 2015). K^+ is the most abundant key cation in almost all organisms, and it plays a fundamental role in basic plant physiological processes (Adams and Shin, 2014). Ca^{2+} is also an essential nutrient for plants; it has a vital structural role in the cell wall and the maintenance of membrane integrity (Bose et al., 2011). Maintaining ion homeostasis through ion uptake and compartmentalization in the root system is crucial for normal plant growth and also for growth during stress (Gupta and Huang, 2014). In addition to the meristem zone, the elongation zone and the mature zone are also important regions of the rhizosphere. The maintenance of appropriate ion concentrations in the elongation and mature zones is important for the growth of plant roots (Sobol and Kordyum, 2009; Assaha et al., 2017; Hu et al., 2020). It is therefore important to understand whether a new rootstock with strong ARD resistance can also maintain the stability of the ion currents in each rhizosphere zone. In addition, new rootstocks should also be able to resist infection by the main harmful *Fusarium* spp. in the soil in China. *Fusarium* spp. and other soilborne fungal complexes responsible for ARD commonly cause root discoloration, root tip necrosis, and/or plant tissue necrosis at the stem base (crown rot) (Marek et al., 2013; Langenhoven et al., 2018). The root systems of highly ARD-resistant rootstocks will exhibit better performance under replant conditions (Leinfelder and Merwin, 2006).

Some crabapples (*Malus spectabilis*) are used as rootstocks for domestic apple production because they contribute beneficial characteristics (Singha, 1989). Through the patented technology of *in situ* breeding (Shen et al., 2015), our research group selected a new elite apple rootstock line named 12-2 that is tolerant to ARD. It is a new line of *M. spectabilis* that has not been identified previously. We initially selected more than 30 ARD-resistant, high-quality lines and planted them in replanted soil with 20-year-old Fuji/*Malus* × *robusta* (CarriŠre) Rehder apples in 2010. By November 2014, only 12-2 and the other superior lines survived, and the trees have continued to survive and grow vigorously to the present day (Gao, 2018; Su et al., 2021). Our previous research and aboveground measurements have shown that ARD has a significant effect on aboveground parameters of T337 and M26 but has no significant effect on 12-2 (Mao et al., 2021a). The root system is the only link connecting the aboveground parts of the plant with the soil, and root development is closely associated with plant ARD tolerance (Wang et al., 2020). Here, we further explore the potential ARD resistance of 12-2 and compare it with the *Malus* rootstocks T337

Abbreviations: ARD, apple replant disease; 12-2, elite apple rootstock line 12-2; MR5, *F. proliferatum* MR5; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; MDA, malondialdehyde; R/S, root-to-shoot ratio; Before-ARD, net rhizoplane ion flow before ARD; ARD-5, net rhizoplane ion flow after immersion in ARD soil extract for 5 min; ARD-30, net rhizoplane ion flow after immersion in ARD soil extract for 30 min.

and M26, which are commonly planted in China. We specifically evaluate three aspects of rootstock performance: (1) changes in net rhizoplane K^+ and Ca^{2+} flow in response to treatment with ARD soil extract for different durations; (2) differences in root physiological parameters between plants grown on replanted and sterilized soil; and (3) differences in tolerance toward infection by the specialized *F. proliferatum* strain MR5. Our work provides useful test materials for the breeding of resistant apple rootstocks, which are important for fundamentally solving the problem of ARD in China.

MATERIALS AND METHODS

Experimental Sites and Materials

The ion current test, pot experiment, and pathogen infection test were conducted at the National Key Seedling Breeding Base of Shandong Agricultural University, Tai'an City, Shandong Province (36.17101°N, 117.16074°E, 134.0 masl). The ion current test and pot experiment were performed from March 2015 to October 2016. The pathogen infection test was performed from August to September 2021. The three test materials were 12-2 (self-named), a tolerant rootstock produced by our group using patented breeding technology, and T337 and M26 tissue culture rootstocks purchased from Shandong Horticultural Techniques and Services Co. Ltd. (Tai'an, Shandong, China). Beginning in early March 2015, tissue cultured seedlings of the three rootstock genotypes were subcultured under the same conditions for 8 months in modified Murashige and Skoog (MS) medium with 30 g L^{-1} sucrose, 7.5 g L^{-1} agar, 0.6 mg L^{-1} 6-BA, and 0.2 mg L^{-1} IBA, pH 5.8. Five explants were placed in each bottle of induction medium and grown in a tissue culture room at 25 ± 2°C with a 16-h light photoperiod and a light intensity of 1000 lx. In early January 2016, tissue culture explants that had been subcultured multiple times were inoculated into rooting medium (modified 1/2 MS medium with 20 g L^{-1} sucrose, 7.5 g L^{-1} agar, 0.2 mg L^{-1} 6-BA, and 1.0 mg L^{-1} IBA, pH 5.8). Five explants were placed in each bottle of induction medium and grown in a tissue culture room at 25 ± 2°C with a 16-h light photoperiod and a light intensity of 1000 lx.

Experimental Treatments

Ion Current Test

On March 3, 2016, three bottles of rooted seedlings of the same size were selected from T337, M26, and 12-2. Each bottle contained two rooted tissue culture seedlings, which were used to measure rhizoplane K^+ and Ca^{2+} ion currents with non-invasive micro-test technology (NMT) (Sun et al., 2009).

Pot Experiment

In early March 2016, rooted plantlets of similar size from each genotype were selected and transplanted into a sterile substrate after hardening off. At the end of March 2016, sixty plants of similar size from each rootstock genotype were selected randomly and transplanted into seedling pots (25 cm diameter, 30 cm depth) with 10 kg of soil; each pot contained three plants. Sixty plants (per genotype) were also randomly divided into two treatment combinations with ten pots (thirty plants) grown in replanted soil, and another ten pots (thirty plants) grown in sterilized soil. A total of sixty pots for the three genotypes (T337, M26, 12-2) were spaced 0.5 × 0.5 m apart at the experimental site. The replanted soil was obtained from a 20-year-old apple orchard in Xuanjiazhuang, Daolang District, Tai'an City, Shandong Province, China. The soil texture was a brown loam. The soil bulk density was 1.31 g cm^{-3}, and its pH was 5.61. The soil nutrient contents included 4.56 mg kg^{-1} ammonium nitrogen, 7.38 mg kg^{-1} nitrate nitrogen, 34.82 mg kg^{-1} available phosphorus, 62.54 mg kg^{-1} available potassium, and 7.92 g kg^{-1} organic matter. The soil was passed through a 10-mesh sieve and mixed well. A portion of the replanted soil was autoclaved at 120°C for 20 min (Zealway Instrument Inc., Xiamen, Fujian, China) and used for the sterilized soil treatment. The pots were irrigated by drip irrigation every 2 days from March to May, once a day from June to September, and every 2 days in October.

Infection Test of Apple Replant Disease-Associated *Fusarium proliferatum* MR5

In mid-August 2021, one hundred seedlings of similar size with 4–5 leaves of T337, M26, and 12-2 were transplanted into black plastic pots (7.0 cm × 5.0 cm × 8.5 cm) filled with sterile substrate after seedling acclimatization. The specialized *F. proliferatum* strain MR5 (MW600437.1) that is associated with ARD has recently been screened and identified (in review); it is highly pathogenic to apple roots and was discovered by the research group of Professor Mao Zhiquan of Shandong Agricultural University. In early September 2021, a layer of pathogenic fungi was inoculated in liquid potato dextrose medium (PDB, Haibo, Qingdao, Shandong, China), cultured for 7 days, and then filtered through eight layers of sterile gauze to obtain a spore suspension. The concentration was measured under a microscope (Nikon Ni-U, Tokyo, Japan) using a hemocytometer (Thermo Fisher Scientific, Waltham, MA, United States), and the final concentration was adjusted to 10^6 spores mL^{-1} with sterile water. On September 8, fifty pots of 12-2, T337, and M26 were irrigated with 20 mL spore suspension, and the other fifty pots were irrigated with an equal volume of PDB medium to serve as controls. The seedlings were grown in a tissue culture room at 24 ± 2°C with a 16-h light photoperiod and a light intensity of 1000 lx. The pots were bottom-irrigated as needed to maintain 60% soil water content.

Measurement Indices

For each experiment (the ion current test, the pot experiment, and the pathogen infection test), there were three rootstock genotypes and a total of six treatment combinations. For each treatment combination, three bottles (two rooted tissue culture seedlings in one bottle were a biological replicate, and there were three biological replicates) containing seedlings of similar growth status were selected randomly for the ion current test. Likewise, three pots per treatment combination (three plants in one pot were a biological replicate, and there were three biological replicates) containing plants of similar growth status were selected randomly for the pot experiment, and three pots (one seedling in one pot was a biological replicate, and there

were three biological replicates) of similar size and growth status were randomly selected for use in each measurement during the pathogen infection test. For all measurements, three technical replicates were performed for each biological replicate. The plants used in the ion current test were harvested on March 3, 2016. The pot experiment was harvested on October 10, 2016 after plants had grown in sterilized or replanted soil for 6 months. The measured root parameters were root architecture, root fresh and dry weights, root metabolic activity, root antioxidant enzyme activities (SOD, POD, CAT), and malondialdehyde (MDA) content. The pathogen infection test was harvested on September 13, 2021. The measured root parameters were root pathological conditions, root architecture, root fresh and dry weights, root metabolic activity, root antioxidant enzyme activities (SOD, POD, CAT), malondialdehyde (MDA) content, root reactive oxygen species levels, and root proline and soluble sugar contents.

Ion Currents

The roots were washed before testing, then placed in the balance solution. The solution used for the two ion measurements was the same: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.2 mM Na_2SO_4, and 0.3 mM MES. Roots were maintained in this solution for 30 min to measure the net rhizoplane ion flow before exposure to ARD (Before-ARD). The roots were then immersed in an ARD soil extract for 5 min. The extract had been prepared by mixing ARD soil with deionized water at a mass ratio of 1:1, extracting for 24 h with shaking, then filtering and storing at 4°C for later use. After equilibrating in the balance solution for 30 min, the net rhizoplane ion flow was measured after a 5-m immersion in ARD soil extract (ARD-5). Finally, the roots were immersed in ARD soil extract for 30 min. After equilibrating in the balance solution for 30 min, the net rhizoplane ion flow was measured after a 30-m immersion in ARD soil extract (ARD-30). The whole root tip was used for these measurements, and ion currents were measured 400 μm from the root tip (meristem) (Ma et al., 2018), 1000 μm from the root tip (elongation zone) (Sun et al., 2009), and 4200 μm from the root tip (mature zone) (Li and Zhang, 2014). Three root samples from each genotype were used for each measurement type (before-ARD, ARD-5, and ARD-30) and ion (Ca^{2+} or K^+). The NMT procedure was performed as described in Sun et al. (2009) with an SIET system (BIO-001A, Younger United States, MA, United States) and MageFlux data analysis software (Xuyue Science and Technology Co., Ltd., Beijing, China).

Root Pathological Conditions

The pathological condition of each root system was observed with a stereomicroscope (Olympus SZX-16, Beijing, Beijing, China).

Root Architecture

Samples were washed with tap water and scanned with an NUScan700 scanner (MICROTEK, Shanghai, China). Total root length, surface area, volume, tip number, and branch number were analyzed with the Delta-T scan image analysis system (Delta-T Devices, Cambridge, United Kingdom).

Root Fresh and Dry Weights

Samples from the pot experiment and the pathogen infection test were weighted fresh and dried after root scanning. The roots and shoots of each plant were weighed separately, then placed in an oven (Suzhou DERIP Oven Manufacturing Co., Ltd., Suzhou, Jiangsu, China) at 115°C for 20 min, and finally dried at 80°C for 24 h. Weights of the dried samples were recorded.

Root Metabolic Activity

Root metabolic activity was measured by the 2,3,5-triphenyltetrazolium chloride (TTC) method (Ruf and Brunner, 2003). Roots were cut into approximately 1-cm pieces, placed in a test tube, and immersed in 5 mL of 0.4% TTC solution and 5 mL of phosphate buffer solution. Two milliliters of 1 M sulfuric acid solution were added to the blank tubes. Tubes were incubated in the dark at 37°C for 1 h, and 2 mL of 1 M sulfuric acid were added to stop the reaction. After standing for 20 min, the roots were gently blotted dry with absorbent paper, placed in a mortar, and ground in 3–4 mL ethyl acetate with a small amount of quartz sand. The red TTF liquid was transferred to a test tube, its volume was adjusted to 10 mL with ethyl acetate, and its absorbance was measured at 485 nm with a spectrophotometer.

Root Antioxidant Enzyme (Superoxide Dismutase, Peroxidase, Catalase) Activities and Malondialdehyde Content

Superoxide dismutase (SOD) activity was measured as described in Zhang et al. (2009). The amount of enzyme required to inhibit 50% of the photochemical reduction of nitroblue tetrazolium (NBT) was defined as one unit of enzyme activity, expressed as U g^{-1} FW^{-1}. Peroxidase (POD) activity was measured by the guaiacol method of Omran (1980) based on the change in absorbance at 470 nm. The amount of enzyme that caused an absorbance change of 0.01 per minute at 470 nm was defined as one unit of enzyme activity, expressed as U g^{-1} FW^{-1} min^{-1}. Catalase (CAT) activity was measured according to the method of Singh et al. (2010) based on the change in absorbance at 240 nm. The amount of enzyme that reduced the absorbance at 240 nm by 0.1 per minute was defined as one unit of enzyme activity, expressed as U g^{-1} FW^{-1} min^{-1}. Malondialdehyde content was measured by the thiobarbituric acid (TBA) method (Lykkesfeldt, 2001). In brief, 1 mL of supernatant was placed in a test tube, 2 mL of 0.67% TBA were added, and the mixture was heated in a boiling water bath for 15 min, then quickly placed in ice water to cool. Absorbance was measured at 600, 532, and 450 nm, and the MDA concentration was calculated as MDA (μ mol g^{-1} FW^{-1}) = 0.1548 (A_{532} – A_{600}) – $0.01344A_{450}$.

Root Reactive Oxygen Species Levels

Root H_2O_2 content and O_2^- production rate were measured using the methods of Bai et al. (2009). The H_2O_2 content was measured by ultraviolet spectrophotometry. Fresh tissue samples (2 g) were combined with 4°C pre-cooled acetone and a small amount of quartz sand at a ratio of 1:1 between the material and the solvent, ground into a homogenate,

and centrifuged at 300 rpm for 10 min. A 1-mL sample of supernatant was combined with 0.1 mL 5% titanium sulfate and 0.2 mL concentrated ammonia water, then centrifuged at 5000 rpm for 10 min. The pellet was washed 3–5 times with acetone until the plant pigments had been removed; then 5 mL of 2 M sulfuric acid was added to the precipitate. When the precipitate was completely dissolved, the absorbance of the titanium peroxide complex was measured at 415 nm and compared to a standard curve to determine the H_2O_2 content.

O_2^- production was measured by the hydroxylamine reaction. Fresh tissue samples (2 g) were combined with 1 mL 0.05 M phosphate buffer (pH 7.8) and ground in an ice bath. The mixture was centrifuged at 12000 rpm for 15 min, and 0.5 mL phosphate buffer and 1 mL 10 M hydroxylamine hydrochloride were added to 0.5 mL of the supernatant. The mixture was allowed to stand at 25°C for 1 h. Then 1 mL 17 mM P-sulfanilic acid and 1 mL 7 mM α-zeamine were added, and the mixture was allowed to stand for 20 min at 25°C. Its absorbance was measured at 530 nm and compared to a standard curve to determine O_2^- production rate.

Root Proline and Soluble Sugar Contents

The proline content was determined by the ninhydrin colorometric method (Wang et al., 2019b). Each fresh tissue sample (0.5 g) was placed into a large test tube, combined with 5 mL 3% sulfosalicylic acid, and placed into a boiling water bath. The tube was removed from the water bath after 10 min (having been stirred evenly during the process) and filtered into a clean test tube after cooling. A 2-mL sample of the extract was placed into another clean test tube with a stopper, and 2 mL glabraic acid and 2 mL acid ninhydrin reagent were added. The mixture was heated in a water bath for 30 min and became red. After cooling, 4 mL toluene was added, and the mixture was shaken for 30 s. After standing, the upper layer was removed and placed into a 10-mL centrifuge tube, then centrifuged at 3000 rpm for 5 min. The upper toluene solution containing proline that had turned red was pipetted into a cuvette, its absorbance was measured at 520 nm with an ultraviolet spectrophotometer, and its proline content was determined by comparison with a standard curve. Toluene was used as the blank control.

The soluble sugar content was determined by the phenol-sulfuric acid method (Masuko et al., 2005). A 0.2-g sample of fresh tissue was chopped and mixed, divided among three graduated test tubes to create three technical replicates, combined with 5 mL of distilled water, sealed with plastic film, and extracted in boiling water for 30 min. The resulting extract was filtered into a 25-mL volumetric flask and brought up to a constant volume (i.e., to the flask marking). A 0.5-mL sample of the liquid was pipetted into a test tube and combined sequentially with 1.5 mL distilled water, 1 mL 9% phenol solution, and 5 mL concentrated sulfuric acid. The sample was mixed to spread the color evenly, its absorbance was measured at 480 nm, and its soluble sugar content was determined by comparison to a standard curve.

Statistical Analyses

The data were analyzed using SPSS (version 17, IBM SPSS, Chicago, IL, United States). One biological replicate value for each treatment combination was the average of two rooted tissue culture seedlings in one bottle for the ion current test, three plants in one pot for the pot experiment, and one seedling in one pot for the pathogen infection test. Unless otherwise noted, the significance of differences among treatment means was assessed by Student's *t*-test and Duncan's multiple range test (DMRT) at a 0.05 probability level.

RESULTS AND ANALYSIS

Rhizoplane Ion Currents

Rhizoplane K^+ Ion Currents

Meristem Zone

In the Before-ARD samples, the root meristem zones (400 μm from the root tip) of T337, M26, and 12-2 all absorbed K^+ from the rhizoplane (**Table 1** and **Supplementary Figure S1**). When roots were exposed to ARD soil extract for 5 min (ARD-5), the average net K^+ fluxes from the rhizoplane into T337 and M26 root meristems were significantly reduced, and 12-2 meristems changed from net K^+ absorption to net K^+ release. The average net K^+ flux into T337 meristems increased significantly in the ARD-30 treatment relative to the ARD-5 treatment, whereas the average net K^+ flux into M26 meristems decreased significantly. The 12-2 roots changed from releasing K^+ in the ARD-5 treatment to absorbing K^+ in the ARD-30 treatment. The average net K^+ flux into 12-2 meristems was significantly higher in the ARD-30 treatment than in the Before-ARD treatment.

Elongation Zone

Before exposure to ARD soil extract, the root elongation zones of all three rootstocks absorbed K^+, consistent with the results from the meristem zones (**Table 2** and **Supplementary Figure S2**). The net K^+ flux in the T337 elongation zone did not differ significantly between Before-ARD and ARD-5, whereas the net K^+ flux in the M26 elongation zone was significantly lower in ARD-5, and that of 12-2 changed from absorption to release. The net K^+ flux in T337 elongation zones did not differ significantly between ARD-5 and ARD-30, whereas the K^+ flux of M26 changed from absorption to release. The K^+ flux of 12-2 changed

TABLE 1 | Effects of ARD on net K^+ flux (pmol cm^{-2} s^{-1}) of three rootstocks in the meristem zone (400 μm from the root tip).

Treatment	T337	M26	12-2
Before-ARD	–74.93 ± 2.17c	–144.38 ± 0.82c	–82.19 ± 3.33b
ARD-5	–46.28 ± 2.76a	–95.75 ± 1.06b	59.70 ± 6.24a
ARD-30	–65.59 ± 1.52b	–36.33 ± 1.49a	–84.63 ± 9.22c

*Before-ARD, net rhizoplane ion flow before ARD treatment; ARD-5, net rhizoplane ion flow after immersion in ARD soil extract for 5 min; ARD-30, net rhizoplane ion flow after immersion in ARD soil extract for 30 min. A negative flow rate indicates that the measured cations are flowing into the root, i.e., the roots are absorbing the ions. Different letters show significant differences ($P < 0.05$). The same conventions are used in **Tables 2–6**, below.*

back from release to absorption in ARD-30 and was significantly higher than in Before-ARD.

Mature Zone

K^+ flux data (**Table 3** and **Supplementary Figure S3**) showed that the mature zones of T337 and M26 absorbed K^+ before and after exposure to ARD soil extract, whereas 12-2 mature zones released K^+. The net K^+ flux into T337 mature root zones was significantly higher in ARD-5 than in Before-ARD; the net K^+ flux into M26 mature root zones was significantly lower; and the net K^+ flux out of 12-2 mature root zones was significantly higher. In the ARD-30 treatment relative to ARD-5, the net K^+ fluxes into T337 and M26 and the net K^+ flux out of 12-2 were significantly reduced.

Rhizoplane Ca^{2+} Ion Currents

Meristem Zone

Before and after exposure to ARD soil extract, the meristems of all three rootstocks showed net Ca^{2+} release (**Table 4** and **Supplementary Figure S4**). After exposure to ARD soil extract for 5 min, the net Ca^{2+} efflux of T337 and 12-2 showed little change, and the net Ca^{2+} flux from M26 was significantly increased. After 30 min of ARD soil extract exposure, the net efflux of Ca^{2+} from T337 and M26 meristem zones was significantly increased. By contrast, net Ca^{2+} efflux from the 12-2 meristem zone was basically unchanged after 30 min of exposure.

Elongation Zone

Before exposure to ARD soil extract, the elongation zone of T337 took up Ca^{2+} from the rhizoplane; by contrast, the elongation zones of M26 and 12-2 showed net Ca^{2+}release (**Table 5** and **Supplementary Figure S5**). After exposure to ARD soil extract for 5 min, T337 elongation zones switched from net Ca^{2+} influx to net Ca^{2+} efflux, and the Ca^{2+} efflux rates of M26 and 12-2 were significantly higher in ARD-5 than in Before-ARD. After 30 min of ARD soil extract exposure, Ca^{2+} efflux from T337 and M26 elongation zones was significantly enhanced relative to ARD-5, and this enhancement was greater in M26. By contrast, Ca^{2+} efflux of 12-2 was significantly lower in ARD-30 than in ARD-5 or Before-ARD.

TABLE 2 | Effects of ARD on net K^+ flux (pmol cm^{-2} s^{-1}) of three rootstocks in the elongation zone (1000 μm from the tip).

Treatment	T337	M26	12-2
Before-ARD	–47.96 ± 1.25b	–103.26 ± 1.05c	–15.80 ± 2.55b
ARD-5	–45.74 ± 1.87ab	–74.43 ± 1.08b	126.11 ± 8.22a
ARD-30	–42.84 ± 1.53a	5.99 ± 4.19a	–52.84 ± 9.23c

TABLE 3 | Effects of ARD on net K^+ flux (pmol cm^{-2} s^{-1}) of three rootstocks in the mature zone (4200 μm from the tip).

Treatment	T337	M26	12-2
Before-ARD	–38.74 ± 1.43b	–83.93 ± 1.21c	23.92 ± 4.91c
ARD-5	–54.74 ± 3.99c	–47.02 ± 1.35b	99.42 ± 7.69a
ARD-30	–33.71 ± 4.21a	–20.77 ± 1.55a	57.08 ± 8.04b

TABLE 4 | Effects of ARD on net Ca^{2+} flux (pmol cm^{-2} s^{-1}) of three rootstocks in the meristem zone (400 μm from the tip).

Treatment	T337	M26	12-2
Before-ARD	53.81 ± 3.26b	28.75 ± 2.58c	68.18 ± 3.15b
ARD-5	52.87 ± 2.42b	37.25 ± 4.14b	71.16 ± 2.29b
ARD-30	109.18 ± 4.35a	195.03 ± 7.65a	79.86 ± 3.12a

TABLE 5 | Effects of ARD on net Ca^{2+} flux (pmol cm^{-2} s^{-1}) of three rootstocks in the elongation zone (1000 μm from the tip).

Treatment	T337	M26	12-2
Before-ARD	–47.37 ± 3.08c	37.30 ± 5.27c	44.79 ± 2.83b
ARD-5	55.74 ± 3.32b	86.17 ± 4.15b	75.05 ± 4.72a
ARD-30	60.78 ± 3.07a	108.30 ± 5.09a	20.90 ± 3.16c

TABLE 6 | Effects of ARD on net Ca^{2+} flux (pmol cm^{-2} s^{-1}) of three rootstocks in the mature zone (4200 μm from the tip).

Treatment	T337	M26	12-2
Before-ARD	45.06 ± 4.15b	138.83 ± 8.01a	34.17 ± 2.05b
ARD-5	35.99 ± 6.73c	46.12 ± 4.96b	44.07 ± 3.37a
ARD-30	98.70 ± 2.52a	136.50 ± 5.27a	32.83 ± 2.86b

Mature Zone

Before and after exposure to ARD soil extract, the meristems of all three rootstocks showed net Ca^{2+} release (**Table 6** and **Supplementary Figure S6**). After exposure to ARD soil extract for 5 min, the net Ca^{2+} fluxes of T337 and M26 mature zones were significantly lower, and this decrease was greater in M26 than in T337. The net Ca^{2+} flux of 12-2 was significantly higher after 5 min of exposure. The net Ca^{2+} efflux of T337 and M26 mature zones was greater after 30 min of exposure than after 5 min of exposure, whereas that of 12-2 mature zones was significantly lower. Compared with Before-ARD, the average Ca^{2+} efflux of T337 was significantly higher in ARD-30, whereas the average Ca^{2+} efflux of M26 and 12-2 did not differ significantly between Before-ARD and ARD-30.

Pot Experiment

Root Architecture

Growth on ARD soil influenced the root architecture of T337 and M26 (**Table 7**): ARD soil significantly reduced root system length, surface area, volume, tip number, and bifurcation number in T337 and significantly reduced the root surface area and volume of M26. ARD soil had no significant effect on root architectural parameters of 12-2.

Root Fresh and Dry Weights

Growth on ARD soil significantly affected the above- and belowground fresh and dry weights of T337 and M26, and it had a significant effect on the root-to-shoot ratio of T337 (**Table 8**). There were no significant effects of ARD soil on fresh or dry weights of 12-2.

TABLE 7 | Effects of ARD soil on the root characteristics of 12-2, T337, and M26 apple rootstocks.

Treatment		Root length (cm)	Root area (cm^2)	Root volume (cm^3)	Root tips number	Root forks number	Root diameter (mm)
T337	Replanted soil	2531.22 ± 186.18	655.15 ± 52.83	13.50 ± 1.23	6553.33 ± 467.32	14380.33 ± 1324.18	0.82 ± 0.01
	Sterilized soil	4363.10 ± 18.51*	1225.43 ± 67.45*	27.61 ± 3.41*	12297.00 ± 937.54*	27376.33 ± 1754.65*	0.96 ± 0.07
M26	Replanted soil	2865.20 ± 345.76	688.14 ± 45.73	13.26 ± 0.15	8170.00 ± 963.28	14683.00 ± 1469.35	0.80 ± 0.07
	Sterilized soil	3462.33 ± 337.52	1031.53 ± 53.17*	24.58 ± 0.53*	8771.00 ± 515.36	20465.33 ± 2263.27	0.89 ± 0.08
12-2	Replanted soil	6006.08 ± 728.38	2132.55 ± 42.17	64.09 ± 8.71	18216.00 ± 1875.35	41602.00 ± 4548.34	1.08 ± 0.14
	Sterilized soil	6767.66 ± 538.23	2234.26 ± 127.68	60.12 ± 3.24	20664.33 ± 2684.36	45094.00 ± 4835.11	1.07 ± 0.03

*Within a rootstock, Student's t-test was used to determine the significance of the difference between control and ARD soil. Data are expressed as mean ± SE. *$p < 0.05$; **$p < 0.01$.*

TABLE 8 | Effects of ARD on the fresh and dry weights of 12-2, T337, and M26 apple rootstocks.

Treatment		Fresh weight (g)		Dry weight (g)		R/S
		Root	Branch	Root	Branch	
T337	Replanted soil	8.09 ± 1.38	14.14 ± 2.18	2.80 ± 0.56	7.39 ± 1.04	0.38 ± 0.03
	Sterilized soil	20.52 ± 0.63*	20.62 ± 1.68*	6.77 ± 0.22**	10.43 ± 0.61*	0.65 ± 0.02**
M26	Replanted soil	14.52 ± 2.27	10.01 ± 1.32	5.53 ± 0.87	5.24 ± 0.77	0.99 ± 0.06
	Sterilized soil	30.13 ± 1.31*	22.99 ± 0.66*	12.32 ± 0.49*	12.41 ± 0.42**	1.01 ± 0.01
12-2	Replanted soil	43.51 ± 2.74	19.66 ± 1.78	15.50 ± 1.32	9.74 ± 0.68	1.60 ± 0.13
	Sterilized soil	46.95 ± 3.61	22.25 ± 1.53	16.82 ± 1.39	11.37 ± 0.82	1.48 ± 0.02

*Within a rootstock, Student's t-test was used to determine the significance of the difference between control and ARD soil. Data are expressed as mean ± SE. *$p < 0.05$; **$p < 0.01$. R/S indicates the root-to-shoot ratio. It is the value obtained by dividing the root dry weight by the shoot dry weight.*

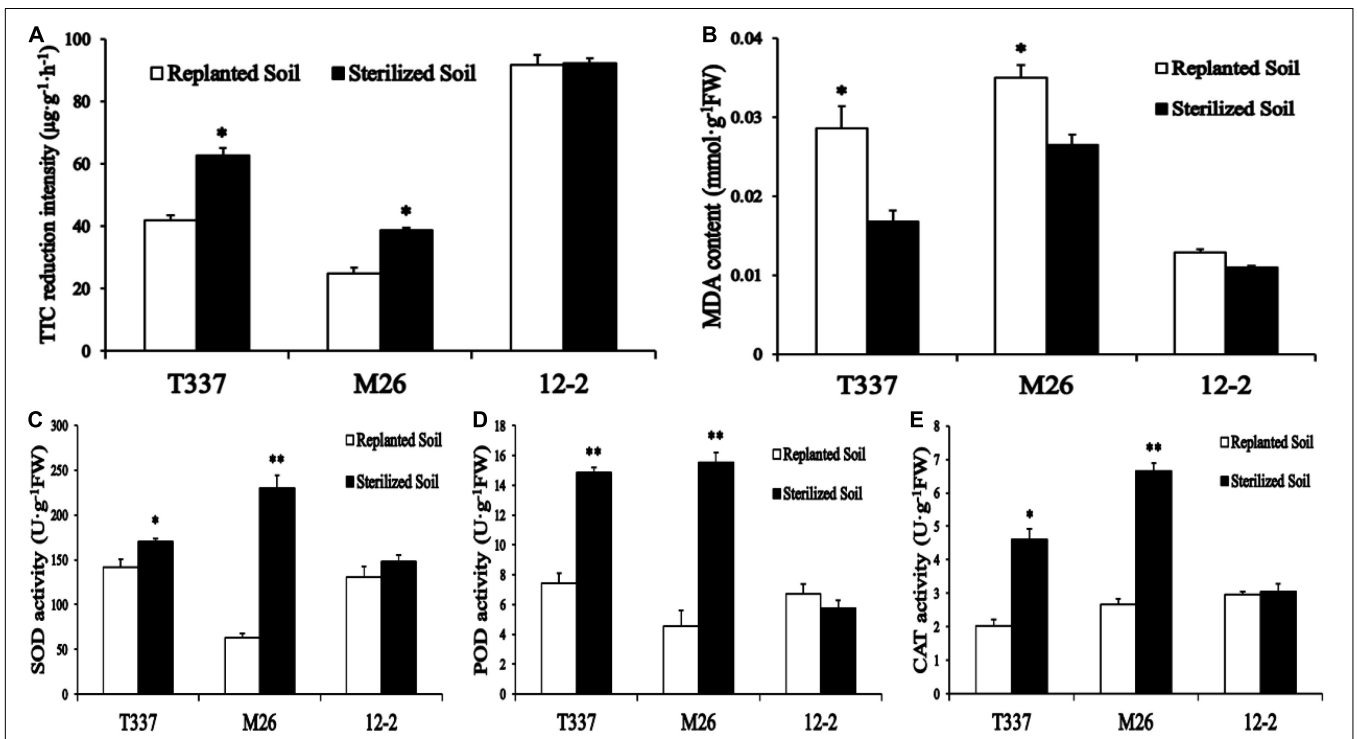

FIGURE 1 | Effects of ARD soil on multiple root parameters of 12-2, T337, and M26 apple rootstocks. **(A)** Root metabolic activity was measured by the 2,3,5-triphenyltetrazolium chloride (TTC) method; **(B)** Root malondialdehyde (MDA) content; **(C)** Root superoxide dismutase (SOD) activity; **(D)** Root peroxidase (POD) activity; **(E)** Root catalase (CAT) activity. Within a rootstock, Student's *t*-test was used to determine the significance of the difference between control and ARD soil. *$p < 0.05$; **$p < 0.01$.

Root Metabolic Activity Measurements

Growth on ARD soil had a significant effect on the root metabolic activity of T337 and M26 (**Figure 1A**) but not on the root metabolic activity of 12-2. The root metabolic activity of 12-2 in replanted soil was also higher than that of T337 and M26.

Root Antioxidant Enzyme Activity and Malondialdehyde Content

Growth on ARD soil altered the antioxidant enzyme activity and MDA content of T337 and M26 roots (**Figures 1B–E**). ARD soil significantly reduced POD and CAT activities in T337 and the antioxidant enzyme activity of M26; it significantly increased the MDA contents of T337 and M26. ARD soil had no significant effect on the antioxidant enzyme activity or MDA content of 12-2.

Infection Test With Apple Replant Disease-Associated *Fusarium proliferatum* MR5

Root Pathological Conditions

As shown in **Figure 2**, the roots of T337, M26, and 12-2 tissue culture seedlings all showed varying degrees of damage 7 d after inoculation with MR5 spore solution. The root color changed to reddish brown (**Figure 2A**), dark gray (**Figure 2B**), or slightly red (**Figure 2C**), whereas the roots of tissue culture seedlings inoculated with PDB solution were bright yellow-brown (**Figures 2D–F**). The root systems of M26 showed the most severe damage, and some roots were even broken and rotted (**Figures 2B,E**). By contrast, the root systems of treated 12-2 plants showed no significant differences from those of control plants (**Figures 2C,F**). T337 showed an intermediate level of root system damage, and there were no signs of decay (**Figures 2A,D**). Under the stereomicroscope, the root systems inoculated with PDB solution appeared smooth, with no damaged spots and a brighter color (**Figure 2G**). After inoculation with MR5 spore solution, the roots of 12-2 appeared mildly injured with slight red spots (**Figure 2H**). The roots of T337 were darkened, the damaged portions shrank, and reddish-brown spots appeared (**Figures 2J,K**); the lateral roots were more severely damaged, displaying atrophy and necrosis (**Figure 2I**). M26 showed the most serious root damage: its roots became black with black spots and bulges on the surface (**Figure 2L**); the root system appeared broken, and hairy hyphae appeared (**Figures 2M,N**).

Root Architecture

Growth with MR5 spore solution did not affect the root architecture of T337, M26, or 12-2 (**Table 9**). MR5 had no effects on the root system length, surface area, volume, tip number, fork number, or diameter of T337, M26, and 12-2.

Root Fresh and Dry Weights

Growth with MR5 spore solution did not affect the above- and belowground fresh and dry weights of T337, M26, and 12-2 (**Table 10**), and it had no significant effect on their root-to-shoot ratios.

Root Metabolic Activity Measurements

Growth with MR5 spore solution had a significant effect on the root metabolic activity of T337 and M26 (**Figure 3A**) but not on that of 12-2. Compared with their respective controls, the root metabolic activity of T337 and M26 decreased by 70.08 and 82.40%, respectively.

Root Antioxidant Enzyme Activity and Malondialdehyde Content

Growth with MR5 spore solution altered the antioxidant enzyme activity and MDA content of T337 and M26 roots (**Figures 3B–E**). MR5 significantly reduced SOD and POD activities in T337, significantly reduced the antioxidant enzyme activity of M26, and significantly increased the MDA contents of T337 and M26. MR5 had no significant effect on the antioxidant enzyme activity or MDA content of 12-2.

Root Reactive Oxygen Species Levels

Growth with MR5 spore solution increased H_2O_2 content and O_2^- production rate of T337 and M26 roots (**Figures 3F,G**). Compared with controls, the H_2O_2 content and O_2^- production rate of MR5-treated T337 roots increased by 44.84 and 48.82%, respectively, and those of M26 roots increased by 35.57 and 76.77%. MR5 had no significant effect on the reactive oxygen species levels of 12-2 roots.

Root Proline and Soluble Sugar Contents

Growth with MR5 spore solution significantly increased the proline and soluble sugar contents of T337 and M26 roots (**Figures 3H,I**). Compared with controls, the proline and soluble sugar contents of MR5-treated T337 roots increased by 33.76 and 22.43%, respectively, and those of M26 roots increased by 112.22 and 35.10%. MR5 had no significant effect on the root proline and soluble sugar contents of 12-2.

DISCUSSION

K^+ can enhance the stress resistance of plants (Kenis and Keulemans, 2007), and the root system is the link between the plant and the soil. Meristem cells are sensitive, and the ion currents of the root system change rapidly and are closely related to plant stress resistance (Newman, 2001). Here, when the meristematic zones of T337, M26, and 12-2 roots were exposed to ARD soil extracts for 30 min, net K^+ flux in the rhizoplane of M26 continued to decrease significantly, whereas 12-2 and T337 were able to recover K^+ absorption to some extent, and the recovery of 12-2 was stronger than that of T337 (**Supplementary Figure S1**). It may be that under stress, 12-2 maintained intracellular K^+ homeostasis by mobilizing K^+ ions in the vacuole and other pools in root cells (Pinto and Ferreira, 2015; Zhang et al., 2020), activating the auxin pool in the endoplasmic reticulum to maintain normal root growth and development to deal with the damage caused by ARD (Friml and Jones, 2010). After 30 min of exposure, the ability of 12-2 to recover net K^+ absorption in the elongation zone was also better than that of T337 and M26 (**Supplementary Figure S2**). 12-2

FIGURE 2 | Root scans and pathology. **(A)** Infected T337; **(B)** Infected M26; **(C)** Infected 12-2; **(D)** Control T337; **(E)** Control M26; **(F)** Control 12-2; **(G)** The microscopic morphology of control PDB-treated 12-2 after 7 days. Control T337 and M26 were similar in appearance to control 12-2; **(H)** The microscopic morphology of 12-2 7 days after inoculation with *Fusarium proliferatum* MR5; **(I–K)** The microscopic morphology of T337 7 days after inoculation with *Fusarium proliferatum* MR5; **(L–N)** The microscopic morphology of M26 7 days after inoculation with *Fusarium proliferatum* MR5. A scale bar is shown in the figure.

may therefore have maintained the turgor pressure and root cell expansion required by cells during root elongation by restoring the absorption of K^+ (Dolan and Davies, 2004; Hu et al., 2020), thereby promoting root elongation, increasing root volume, and quickly responding to stress (Li et al., 2011). In the root mature zone of 12-2, net K^+ influx increased significantly after exposure

TABLE 9 | Effects of *Fusarium proliferatum* MR5 on the root characteristics of 12-2, T337, and M26 apple rootstocks.

Treatment		Root length (mm)	Root area (mm^2)	Root Volume (mm^3)	Root tips number	Root forks number	Root diameter (mm/10)
T337	Infected	1483.96 ± 48.63	1877.42 ± 196.84	202.12 ± 17.67	250.33 ± 25.21	1093.00 ± 123.56	0.44 ± 0.02
	Control	1404.88 ± 59.87	1648.07 ± 67.53	183.79 ± 14.50	223.67 ± 9.02	972.33 ± 37.32	0.43 ± 0.01
M26	Infected	1325.48 ± 37.65	1606.91 ± 120.38	172.18 ± 24.40	210.33 ± 31.32	854.33 ± 73.99	0.38 ± 0.03
	Control	1320.16 ± 69.66	1611.64 ± 164.24	172.99 ± 25.69	331.67 ± 43.32	977.67 ± 28.32	0.38 ± 0.02
12-2	Infected	1520.71 ± 149.37	2122.73 ± 118.28	237.61 ± 14.92	347.00 ± 63.66	1111.67 ± 134.46	0.45 ± 0.03
	Control	1566.66 ± 66.24	2189.93 ± 198.57	245.25 ± 34.74	335.67 ± 32.74	1159.00 ± 73.82	0.44 ± 0.01

*Within a rootstock, Student's t-test was used to determine the significance of the difference between control and ARD soil. Data are expressed as mean ± SE. *p < 0.05; **p < 0.01.*

TABLE 10 | Effects of *Fusarium proliferatum* MR5 on the fresh and dry weights of 12-2, T337, and M26 apple rootstocks.

Treatment		Fresh weight (g)		Dry weight (g)		R/S
		Root	Branch	Root	Branch	
T337	Infected	0.26 ± 0.01	0.62 ± 0.01	0.05 ± 0.00	0.26 ± 0.01	0.20 ± 0.01
	Control	0.27 ± 0.01	0.65 ± 0.01	0.05 ± 0.00	0.26 ± 0.01	0.19 ± 0.01
M26	Infected	0.18 ± 0.00	0.54 ± 0.02	0.04 ± 0.00	0.16 ± 0.00	0.25 ± 0.01
	Control	0.18 ± 0.01	0.55 ± 0.01	0.04 ± 0.00	0.17 ± 0.01	0.24 ± 0.01
12-2	Infected	0.27 ± 0.00	0.78 ± 0.01	0.07 ± 0.00	0.30 ± 0.01	0.22 ± 0.02
	Control	0.27 ± 0.00	0.78 ± 0.02	0.06 ± 0.00	0.29 ± 0.02	0.22 ± 0.01

*Within a rootstock, Student's t-test was used to determine the significance of the difference between control and ARD soil. Data are expressed as mean ± SE. *p < 0.05; **p < 0.01. R/S indicates the root-to-shoot ratio. It is the value obtained by dividing the root dry weight by the shoot dry weight.*

to ARD extract for 5 min, but it decreased significantly after 30 min, consistent with the study of Wang H. Y. et al. (2018) (**Supplementary Figure S3**). After 5 min of exposure, ARD may have induced a rapid increase in H_2O_2 in 12-2 root cells, and various root K^+ osmotic channels are known to be activated by H_2O_2 (Wang H. Y. et al., 2018). H_2O_2 produced in roots can interact with transition metals (Fe^{2+} or Cu^{2+}) to produce highly reactive OH·, thereby activating various K^+ permeation channels and causing large amounts of K^+ efflux (Shabala and Pottosin, 2014). After 30 min of exposure, the rapid accumulation of H_2O_2 in the mature zone of 12-2 roots may induce plant resistance. The subsequent removal of H_2O_2 may have induced the closure of K^+ permeation channels, thereby reducing K^+ outflow (Pinto and Ferreira, 2015; Wang H. Y. et al., 2018). On the other hand, the K^+ accumulated in the root maturation zone may be transported to other root cells over time (Zhang et al., 2020), maintaining K^+ homeostasis and thereby enhancing stress resistance (Pinto and Ferreira, 2015; Assaha et al., 2017). However, ARD affected K^+ absorption of T337 and M26 mature root zones, causing a decrease in net K^+ influx (Liu et al., 2012). Therefore, it appears that the 12-2 root system can quickly adjust its K^+ homeostasis, maintain a steady state, and enhance ARD resistance.

Ca^{2+} plays an important role in plant stress signal transduction (Xu et al., 2018). In this experiment, ARD-30 caused a significant increase in the net efflux of Ca^{2+} from the root meristems of T337 and M26, whereas the net efflux of Ca^{2+} was basically unchanged in 12-2 (**Supplementary Figure S4**). Substantial Ca^{2+} efflux is not conducive to calcium signal transduction and affects the normal plant stress response (Feng et al., 2016). The relative homeostasis of Ca^{2+} in 12-2 roots may allow for rapid activation or inhibition of various membrane ion channels or specific enzymes, induce the expression of antiretroviral genes, stabilize cell walls and cell membranes, and thereby enhance environmental adaptation and improve ARD resistance (Roelfsema and Hedrich, 2010; Qi and Zhang, 2020). Five minutes of exposure to ARD soil extract had little effect on net efflux of Ca^{2+} in the rhizoplane of the three rootstocks. This may be because short-term ARD-induced Ca^{2+} efflux is mediated by Ca^{2+} channels (Kuster et al., 2011), and the ARD-5 treatment did not reach the specific threshold of Ca^{2+} stress that stimulates the meristem (Bose et al., 2011). With increasing exposure time of the root elongation zone, net Ca^{2+} efflux from 12-2 first increased and then decreased, in contrast to the continuously increasing net efflux from M26 and T337 (**Supplementary Figure S5**). This result suggested that ARD soil extract may weaken the growth of T337 and M26 roots in response to gravity, thereby destroying the steady state of Ca^{2+}. Because 12-2 was less affected, it could maintain root cap gravity perception and the resulting asymmetric growth that occurs during root elongation, thereby quickly restoring steady-state Ca^{2+} concentration (Sobol and Kordyum, 2009; Dodd et al., 2010). These results were consistent with the root physiology and structure measurements, although the specific underlying mechanisms remain to be explored. The change in Ca^{2+} flux in the mature root zone of 12-2 was opposite to that of T337 and M26 (**Supplementary Figure S6**). ARD may therefore have affected the Ca^{2+}/H^+ exchanger and Ca^{2+}-ATPase of T337 and M26. Ca^{2+} release into the cell or into intracellular organelles may have been inhibited, maintaining cellular Ca^{2+} concentrations at a higher level (Bose et al., 2011). Higher concentrations of Ca^{2+} could then trigger

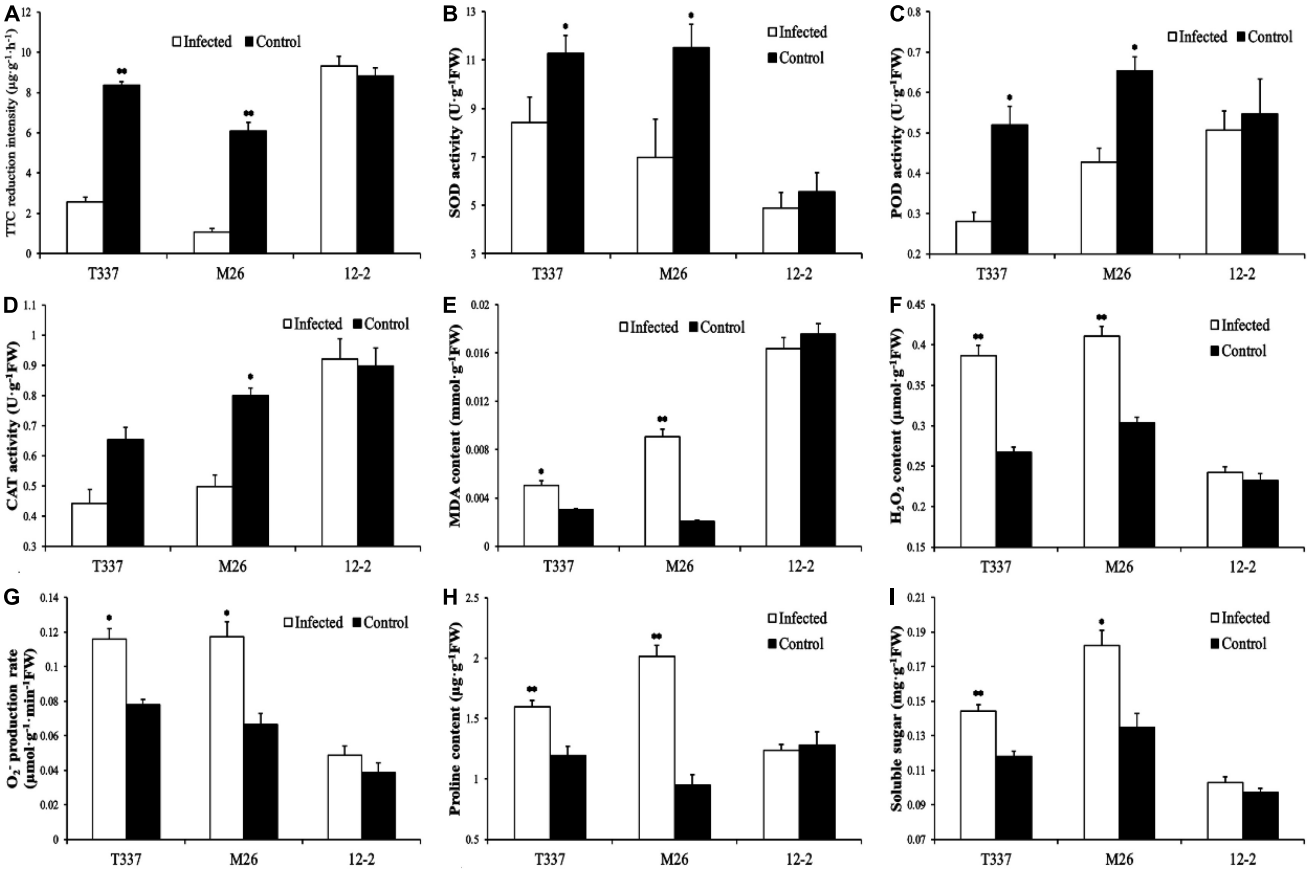

FIGURE 3 | Effects of *Fusarium proliferatum* MR5 on multiple root parameters of 12-2, T337, and M26 apple rootstocks. **(A)** Root metabolic activity measurements; **(B–D)** Root antioxidant enzyme activity; **(E)** Root MDA content; **(F,G)** Root reactive oxygen species level; **(H,I)** Root permeable substance content. Within a rootstock, Student's *t*-test was used to determine the significance of the difference between control and ARD soil. $*p < 0.05$; $**p < 0.01$.

the aggregation of proteins and nucleic acids, resulting in the precipitation of phosphate, which is not conducive to plant health (Case et al., 2007). The mature zones of 12-2 may have had an efficient transport system that reduced Ca^{2+} efflux and maintained the steady state of the microenvironment (Feng et al., 2016). Therefore, we speculate that 12-2 may be able to quickly exchange information with other tissues and organs under ARD, maintaining the stability of Ca^{2+} at a specific time and location and thereby enhancing ARD tolerance.

Changes in root physiological indices in the pot experiment and the *Fusarium* tolerance test also showed that 12-2 had greater tolerance to ARD than the other tested rootstocks. Compared with its untreated control, 12-2 maintained high root growth and a large root surface area in replanted soil, and its root physiological indices differed little from those observed in sterilized soil. This suggested that 12-2 could more quickly perceive stress signals and regulate the expression of stress-related genes through signal transduction pathways, thereby regulating its physiological state and the distribution of metabolites among organs (Lager et al., 2010; Ghonaim et al., 2021) and maintaining the total length and surface area of the root system to cope with stress (Comas et al., 2013; Chen et al., 2020). In addition, maintenance of a high root growth rate in 12-2 may have reduced ARD sensitivity, limited infection by harmful fungi (Emmett et al., 2014), maintained root structure and function (Atucha et al., 2014), reduced root rot and browning (Ghosh and Xu, 2014), and promoted normal growth and increased ARD resistance. Under ARD stress, antioxidant enzyme activities were significantly lower in T337 and M26 roots from replanted soil than in those from sterilized soil, and high levels of MDA accumulated. By contrast, there were no significant differences in antioxidant enzyme activity and MDA content between the two treatments for 12-2 (**Figure 1**). It may be that under ARD, superoxide ($O_2^{\cdot}$) and other harmful substances accumulate in larger amounts in M26 and T337 (Zhang et al., 2013; Berni et al., 2019), exceeding their cellular tolerance (Zhang et al., 2021). Although SOD, POD, and CAT work together to disproportionate $O_2^{\cdot}$ to H_2O_2 and O_2, reducing its toxicity to a certain extent (Sharma et al., 2012; Li et al., 2019), the H_2O_2 produced by disproportionation will accumulate to excessively high levels over time, leading to massive production of the membrane lipid peroxidation product MDA (Gill and Tuteja, 2010). MDA reacts with and denatures cell macromolecules such as proteins and nucleic acids, thereby destroying cell

structure and function (Zhao et al., 2019), rapidly inhibiting cell antioxidant enzyme activity, and ultimately leading to cell death (Ayala et al., 2014; Mu et al., 2021). Therefore, within one week of infection by ARD-associated *F. proliferatum* MR5, the roots of M26 and T337 showed numerous brown spots, rot, and fractures (**Figure 2**). These observations were consistent with previous reports of epidermal cell lysis, disintegration of cortical cells, root tip necrosis, and the almost complete loss of functional root hairs due to excessive accumulation of reactive oxygen species (Petrov et al., 2015; Han et al., 2021). Unlike the other two rootstocks, 12-2 could maintain a degree of balance between ROS production and scavenging, thereby maintaining the normal cellular redox state (Tanveer and Shah, 2017). ROS induce the accumulation of large amounts of lignin and callosin near the host infection site, participate in the oxidative cross-linking of cell wall structural proteins, and produce insoluble dimers and tetramers that are deposited on the cell wall, thereby strengthening it and enhancing the plant's mechanical barrier against pathogens (Sharma et al., 2012; Castro et al., 2021). The significant increase in soluble sugars and proline were also consistent with the poor ARD tolerance of M26 and T337 roots, which led to excessive production of ROS and excessive oxidative damage to lipid membranes (Sharma et al., 2012; Yang et al., 2015); lack of accumulation of these stress-related metabolites in 12-2 provided further evidence that it had improved ARD tolerance.

The results presented here suggest that 12-2 has a certain degree of ARD resistance and tolerance to ARD-associated *F. proliferatum* MR5; nonetheless, the molecular mechanisms by which 12-2 responds to ARD and MR5 are still unknown. *Fusarium* is the main harmful fungus that causes ARD in China, but it is unclear whether 12-2 is also resistant to oomycetes such as *Pythium* and *Phytophthora*. To further assess the performance of 12-2, it will be necessary to perform further comparisons with other elite rootstocks. Many experiments must be performed on new rootstocks, including evaluations of their graft compatibility, fruit yield, and survival and growth in other planting regions. These topics will be the focus of future research.

CONCLUSION

Measurements of K^+ and Ca^{2+} ion currents in the rhizoplane meristem, elongation, and maturation zones suggested that 12-2 exhibited good resistance to ARD in each area of the root. And there were no significant differences in the root physiological parameters of 12-2 rootstock in replanted or sterilized soil. A pathogen infection test also showed that 12-2 had good resistance to ARD-associated *F. proliferatum* MR5. ARD had a greater impact on K^+ and Ca^{2+} ion currents in various root zones of T337 and M26 and on most root physiological parameters. T337 and M26 showed a degree of intolerance to ARD-associated *F. proliferatum* MR5. 12-2 could be used as an important material for the breeding of ARD-resistant apple rootstocks, which will be important for fundamentally solving the problem of ARD in China.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YM and XSh planned and designed the research. YM, YY, XC, HW, XSu, XQ, and YL performed experiments, conducted fieldwork, and analyzed data. YM, YY, YH, and XSh wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by project supported by the National Natural Science Foundation of China, award number 32072520; Fruit Innovation Team Project of Shandong Province (CN), award number SDAIT-06-07; Natural Science Foundation of Shandong Province (CN), award number ZR2020MC132, and Industrialization Project of Improved Varieties in Shandong Province (CN), award number 2019LZGC007.

ACKNOWLEDGMENTS

We thank Xuesen Chen and Zhiquan Mao for the breeding of apple rootstock superior line named 12-2, and Zhiquan Mao for providing the ARD-associated *F. proliferatum* MR5.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.734430/full#supplementary-material

REFERENCES

Adams, E., and Shin, R. (2014). Transport, signaling, and homeostasis of potassium and sodium in plants. *J. Integr. Plant Biol.* 56, 231–249. doi: 10.1111/jipb.12159

Assaha, D. V. M., Ueda, A., Saneoka, H., Al-Yahyai, R., and Yaish, M. W. (2017). The role of Na^+ and K^+ transporters in salt stress adaptation in glycophytes. *Front. Plant Sci.* 8:509. doi: 10.3389/fphys.2017.00509

Atucha, A., Emmett, B., and Bauerle, T. L. (2014). Growth rate of fine root systems influences rootstock tolerance to replant disease. *Plant Soil* 376, 337–346. doi: 10.1007/s11104-013-1977-5

Ayala, A., Muñoz, M. F., and Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid. Med. Cell. Longev.* 2014:360438. doi: 10.1155/2014/360438

Bai, R., Ma, F., Liang, D., and Zhao, X. (2009). Phthalic acid induces oxidative stress and alters the activity of some antioxidant enzymes in roots of *Malus prunifolia*. *J. Chem. Ecol.* 35, 488–494. doi: 10.1007/s10886-009-9615-7

Berni, R., Luyckx, M., Xu, X., Legay, S., Sergeant, K., Hausman, J. F., et al. (2019). Reactive oxygen species and heavy metal stress in plants: impact on the cell wall and secondary metabolism. *Environ. Exp. Bot.* 161, 98–106. doi: 10.1016/j.envexpbot.2018.10.017

Bose, J., Pottosin, I. I., Shabala, S. S., Palmgren, M. G., and Shabala, S. (2011). Calcium efflux systems in stress signaling and adaptation in plants. *Front. Plant Sci.* 2:85. doi: 10.3389/fpls.2011.00085

Bowen, J. K., Mesarich, C. H., Bus, V. G. M., Beresford, R. M., Plummer, K. M., and Templeton, M. D. (2010). Venturia inaequalis: the causal agent of apple scab. *Mol. Plant Pathol.* 12, 105–122. doi: 10.1111/j.1364-3703.2010.00656.x

Case, R. M., Eisner, D., Gurney, A., Jones, O., Muallem, S., and Verkhratsky, A. (2007). Evolution of calcium homeostasis: from birth of the first cell to an omnipresent signalling system. *Cell Calcium* 42, 345–350. doi: 10.1016/j.ceca.2007.05.001

Castro, B., Citterico, M., Kimura, S., Stevens, D. M., Wrzaczek, M., and Coaker, G. (2021). Stress-induced reactive oxygen species compartmentalization, perception and signalling. *Nat. Plants* 7, 403–412. doi: 10.1038/s41477-021-00887-0

Chen, P., Wang, Y. Z., Liu, Q. Z., Zhang, Y. T., Li, X. Y., Li, H. Q., et al. (2020). Phase changes of continuous cropping obstacles in strawberry (*Fragaria* × *ananassa* Duch.) production. *Appl. Soil Ecol.* 155:103626. doi: 10.1016/j.apsoil.2020.103626

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

Dodd, A. N., Kudla, J., and Sanders, D. (2010). The language of calcium signaling. *Annu. Rev. Plant Biol.* 61, 593–620. doi: 10.1146/annurev-arplant-070109-104628

Dolan, L., and Davies, J. (2004). Cell expansion in roots. *Curr. Opin. Plant Biol.* 7, 33–39. doi: 10.1016/j.pbi.2003.11.006

Duan, C. X., Qin, Z. H., Yang, Z. H., Li, W. X., Sun, S. L., Zhu, Z. D., et al. (2016). Identification of pathogenic *Fusarium* spp. causing maize ear rot and potential mycotoxin production in China. *Toxins* 8:186. doi: 10.3390/toxins8060186

Emmett, B., Nelson, E. B., Kessler, A., and Bauerle, T. L. (2014). Fine-root system development and susceptibility to pathogen colonization. *Planta* 239, 325–340. doi: 10.1007/s00425-013-1989-7

Fallahi, E., Colt, W. M., Fallahi, B., and Chun, I. J. (2001). The importance of apple rootstocks on tree growth, yield, fruit quality, leaf nutrition, and photosynthesis with an emphasis on 'Fuji'. *Horttechnology* 12, 38–44. doi: 10.21273/HORTTECH.12.1.38

Feng, X., Liu, W. X., Zeng, F. R., Chen, Z. H., Zhang, G. P., and Wu, F. B. (2016). K^+ Uptake, H^+-ATPase pumping activity and Ca^{2+} efflux mechanism are involved in drought tolerance of barley. *Environ. Exp. Bot.* 129, 57–66. doi: 10.1016/j.envexpbot.2015.11.006

Friml, J., and Jones, A. R. (2010). Endoplasmic reticulum: the rising compartment in auxin biology. *Plant Physiol.* 154, 458–462. doi: 10.1104/pp.110.161380

Gao, F. F. (2018). *The Continuous Cropping Testing and Rapid Propagation of Primary Apple Rootstocks Superior Lines.* Bachelor's thesis. Tai'an: Shandong Agricultural University.

Ghonaim, M. M., Mohamed, H. I., and Orman, A. A. A. (2021). Evaluation of wheat (*Triticum aestivum* L.) salt stress tolerance using physiological parameters and retrotransposon-based markers. *Genet. Resour. Crop Evol.* 68, 227–242. doi: 10.1007/s10722-020-00981-w

Ghosh, D., and Xu, J. (2014). Abiotic stress responses in plant roots: a proteomics perspective. *Front. Plant Sci.* 5:6. doi: 10.3389/fpls.2014.00006

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930. doi: 10.1016/j.plaphy.2010.08.016

Guo, X. J., Han, T. T., Wang, R., Zhang, Z., Ge, R., Mao, Z. Q., et al. (2015). Differences of growth and root absorption of different apple rootstock seedlings in replant stress (In Chinese). *J. Plant Nutr. Fert.* 21, 1312–1319. doi: 10.11674/zwyf.2015.0526

Gupta, B., and Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genomics* 2014:701596. doi: 10.1155/2014/701596

Han, G. L., Li, Y. X., Qiao, Z. Q., Wang, C. F., Zhao, Y., Guo, J. R., et al. (2021). Advances in the regulation of epidermal cell development by C2H2 zinc finger proteins in plants. *Front. Plant Sci.* 12:754512. doi: 10.3389/fpls.2021.754512

Hu, W., Di, Q., Zhang, J., Liu, J., and Shi, X. J. (2020). Response of grafting tobacco to low potassium stress. *BMC Plant Biol.* 20:286. doi: 10.1186/s12870-020-02481-6

Kenis, K., and Keulemans, J. (2007). Study of tree architecture of apple (*Malus* × *domestica* Borkh.) by QTL analysis of growth traits. *Mol. Breed.* 19, 193–208. doi: 10.1007/s11032-006-9022-5

Kuster, G. M., Lancel, S., Zhang, J. M., Communal, C., Trucillo, M. P., Lim, C. C., et al. (2011). Redox-mediated reciprocal regulation of SERCA and Na^+/Ca^{2+}-exchanger contributes to SR Ca^{2+}-depletion in cardiac myocytes. *Free Radic. Biol. Med.* 48, 1182–1187. doi: 10.1016/j.freeradbiomed.2010.01.038

Lager, I., Andréasson, O., Dunbar, T. L., Andreasson, E., Escobar, M. A., and Rasmusson, A. G. (2010). Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. *Plant Cell Environ.* 33, 1513–1528. doi: 10.1111/j.1365-3040.2010.02161.x

Langenhoven, S. D., Halleen, F., Spies, C. F. J., Stempien, E., and Mostert, L. (2018). Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa. *Phytopathol. Mediterr.* 57, 519–537. doi: 10.14601/Phytopathol_Mediterr-23921

Leinfelder, M. M., and Merwin, I. A. (2006). Rootstock selection, replant soil treatments, and tree planting positions as factors in managing apple replant disease. *Hortscience* 41, 394–401. doi: 10.21273/HORTSCI.41.2.394

Li, C. X., Sun, J. S., Li, F. S., Zhou, X. G., Li, Z. Y., Qiang, X. M., et al. (2011). Response of root morphology and distribution in maize to alternate furrow irrigation. *Agric. Water Manage.* 98, 1789–1798. doi: 10.1016/j.agwat.2011.07.005

Li, J. X., Zhou, X., Wei, B. D., Cheng, S. C., Zhou, Q., and Ji, S. J. (2019). GABA application improves the mitochondrial antioxidant system and reduces peel browning in 'Nanguo' pears after removal from cold storage. *Food Chem.* 297:124903. doi: 10.1016/j.foodchem.2019.05.177

Li, P. F., and Zhang, B. (2014). K^+ and Na^+ fluxes in roots of two Chinese Iris populations. *Front. Agric. Sci. Eng.* 2, 144–149. doi: 10.15302/J-FASE-2014016

Liu, B. H., Li, M. J., Cheng, L., and Liang, D. (2012). Influence of rootstock on antioxidant system in leaves and roots of young apple trees in response to drought stress. *Plant Growth Regul.* 67, 247–256. doi: 10.1007/s10725-012-9683-5

Liu, Z. (2013). *Isolation and Identification of Fungi Pathogen from Apple Replant Disease in Bohai Bay Region and the Screening of the Antagonistic Trichoderma Strains.* Bachelor's thesis. Tai'an: Shandong Agricultural University.

Lykkesfeldt, J. (2001). Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: comparison with ultraviolet-visible spectrophotometry. *Clin. Chem.* 47, 1725–1727. doi: 10.1093/clinchem/47.9.1725

Ma, K. S., Shi, W. S., Xu, M. Y., Liu, J. X., and Zhang, F. X. (2018). Genome-wide identification and characterization of long non-coding RNA in wheat roots in response to Ca^{2+} channel blocker. *Front. Plant Sci.* 9:244. doi: 10.3389/fpls.2018.00244

Mao, Y. F., Zhang, L. L., Wang, Y. Y., Yang, L., Yin, Y. J., Su, X. F., et al. (2021b). Effects of polycyclic aromatic hydrocarbons (PAHs) from different sources on soil enzymes and microorganisms of *Malus prunifolia* var. Ringo. *Arch. Agron. Soil Sci.* 67, 2048–2062. doi: 10.1080/03650340.2020.1820488

Mao, Y. F., Yin, Y. J., Cui, X. L., Wang, H. Y., Su, X. F., Qin, X., et al. (2021a). Detection of above-ground physiological indices of an apple rootstock superior line 12-2 with improved Apple Replant Disease (ARD) resistance. *Horticulturae* 7:337. doi: 10.3390/horticulturae7100337

Marek, S. M., Yaghmour, M. A., and Bostock, R. M. (2013). *Fusarium* spp., *Cylindrocarpon* spp., and environmental stress in the etiology of a canker disease of cold-stored fruit and nut tree seedlings in California. *Plant Dis.* 97, 259–270. doi: 10.1094/PDIS-04-12-0355-RE

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S., and Lee, Y. C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal. Biochem.* 339, 69–72. doi: 10.1016/j.ab.2004.12.001

Mazzola, M., and Manici, L. M. (2012). Apple replant disease: role of microbial ecology in cause and control. *Annu. Rev. Phytopathol.* 50, 45–65. doi: 10.1146/annurev-phyto-081211-173005

Mu, J. F., Yang, F. P., Tan, F., Zhou, X. R., Pan, Y. N., Long, X. Y., et al. (2021). Determination of polyphenols in *Ilex kudingcha* and insect tea (Leaves Altered by Animals) by ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-QqQ-MS) and comparison of their anti-aging effects. *Front. Pharmacol.* 11:600219. doi: 10.3389/fphar.2020.600219

Narwal, S. S. (2010). Allelopathy in ecological sustainable organic agriculture. *Allelopathy J.* 25, 51–72.

Newman, I. A. (2001). Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant Cell Environ.* 21, 1–14. doi: 10.1046/j.1365-3040.2001.00661.x

Omran, R. G. (1980). Peroxide levels and the activities of catalase, peroxidase, and indoleacetic acid oxidase during and after chilling cucumber seedlings. *Plant Physiol.* 65, 407–408. doi: 10.1104/pp.65.2.407

Pan, F. B., Xiang, L., Wang, S., Li, J. J., Shen, X., Chen, X. S., et al. (2017). Effects of short-term rotation and *Trichoderma* application on the soil environment and physiological characteristics of *Malus hupehensis* Rehd. seedlings under replant conditions. *Acta Ecol. Sin.* 37, 315–321. doi: 10.1016/j.chnaes.2017.09.003

Petrov, V., Hille, J., Mueller-Roeber, B., and Gechev, T. S. (2015). ROS-mediated abiotic stress-induced programmed cell death in plants. *Front. Plant Sci.* 6:69. doi: 10.3389/fpls.2015.00069

Pinto, E., and Ferreira, I. M. P. L. V. O. (2015). Cation transporters/channels in plants: tools for nutrient biofortification. *J. Plant Physiol.* 179, 64–82. doi: 10.1016/j.jplph.2015.02.010

Qi, F. F., and Zhang, F. X. (2020). Cell cycle regulation in the plant response to stress. *Front. Plant Sci.* 10:1765. doi: 10.3389/fpls.2019.01765

Reim, S., Siewert, C., Winkelmann, T., Wöhner, T., Hanke, M. V., and Flachowsky, H. (2019). Evaluation of *Malus* genetic resources for tolerance to apple replant disease (ARD). *Sci. Hortic.* 256:108517. doi: 10.1016/j.scienta.2019.05.044

Rivard, C. L., O'Connell, S., Peet, M. M., and Welker, R. M. (2012). Grafting tomato to manage bacterial wilt caused by *Ralstonia solanacearum* in the southeastern United States. *Plant Dis.* 96, 973–978. doi: 10.1094/PDIS-12-10-0877

Roelfsema, M. R. G., and Hedrich, R. (2010). Making sense out of Ca^{2+} signals: their role in regulating stomatal movements. *Plant Cell Environ.* 33, 305–321. doi: 10.1111/j.1365-3040.2009.02075.x

Ruf, M., and Brunner, I. (2003). Vitality of tree fine roots: reevaluation of the tetrazolium test. *Tree Physiol.* 23, 257–263. doi: 10.1093/treephys/23.4.257

Rumberger, A., Yao, S. R., Merwin, I. A., Nelson, E. B., and Thies, J. E. (2004). Rootstock genotype and orchard replant position rather than soil fumigation or compost amendment determine tree growth and rhizosphere bacterial community composition in an apple replant soil. *Plant Soil* 264, 247–260. doi: 10.1023/B:PLSO.0000047760.13004.94

Shabala, S., and Pottosin, I. (2014). Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. *Physiol. Plant.* 151, 257–279. doi: 10.1111/ppl.12165

Sharma, P., Jha, A. B., Dubey, R. S., Pessarakli, M., and Polle, A. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot.* 2012:217037. doi: 10.1155/2012/217037

Shen, X., Mao, Z. Q., Ni, W. R., Wang, R., Hu, Y. L., Chen, X. S., et al. (2015). *In situ Breeding method for Three-Stage Selection of Apple Rootstock Tolerant to ARD. China Patent No. CN104488645A. (In Chinese)*. Beijing: China National Intellectual Property Administration.

Singh, B. K., Sharma, S. R., and Singh, B. (2010). Antioxidant enzymes in cabbage: variability and inheritance of superoxide dismutase, peroxidase and catalase. *Sci. Hortic.* 124, 9–13. doi: 10.1016/j.scienta.2009.12.011

Singha, S. (1989). "Crabapple (*Malus* spp.)," in *Trees II. Biotechnology in Agriculture and Forestry*, Vol. 5, ed. Y. P. S. Bajaj (Berlin: Springer). doi: 10.1007/978-3-642-61535-1_3

Sobol, M., and Kordyum, E. (2009). Distribution of calcium ions in cells of the root distal elongation zone under clinorotation. *Micrograv. Sci. Technol.* 21, 179–185. doi: 10.1007/s12217-008-9045-0

Su, X. F., Chai, S. S., Mao, Y. F., Zhang, L. L., Yin, Y. J., Liu, Y. P., et al. (2021). Polyploid induction and identification of apple rootstocks with tolerance to continuous cropping (In Chinese). *Mol. Plant Breed.* 2021, 1–12.

Sun, J., Dai, S. X., Wang, R. G., Chen, S. L., Li, N. Y., Zhou, X. Y., et al. (2009). Calcium mediates root K^+/Na^+ homeostasis in poplar species differing in salt tolerance. *Tree Physiol.* 29, 1175–1186. doi: 10.1093/treephys/tpp048

Tanveer, M., and Shah, A. (2017). An insight into salt stress tolerance mechanisms of *Chenopodium album*. *Environ. Sci. Pollut. Res. Int.* 24, 16531–16535. doi: 10.1007/s11356-017-9337-2

Wang, G. S., Yin, C. M., Pan, F. B., Wang, X. B., Xiang, L., Wang, Y. F., et al. (2018). Analysis of the fungal community in apple replanted soil around Bohai Gulf. *Hortic. Plant J.* 4, 175–181. doi: 10.1016/j.hpj.2018.05.003

Wang, H. Y., Shabala, L., Zhou, M. X., and Shabala, S. (2018). Hydrogen peroxide-induced root Ca^{2+} and K^+ fluxes correlate with salt tolerance in cereals: towards the cell-based phenotyping. *Int. J. Mol. Sci.* 19:702. doi: 10.3390/ijms19030702

Wang, H. Y., Zhao, L., Jiang, W. T., Zhang, R., Chen, R., Mao, Y. F., et al. (2021). Effects of *Allium fistulosum-Brassica juncea-Triticum aestivum* rotation a year on the soil microbial environment and the subsequent growth of young apple trees. *Sci. Hortic.* 290:110549. doi: 10.1016/j.scienta.2021.110549

Wang, N. Q., Kong, C. H., Wang, P., and Meiners, S. J. (2020). Root exudate signals in plant-plant interactions. *Plant Cell Environ.* 44, 1044–1058. doi: 10.1111/pce.13892

Wang, Y., Li, W., Xu, X. F., Qiu, C. P., Wu, T., Wei, Q. P., et al. (2019a). Progress of apple rootstock breeding and its use. *Hortc. Plant J.* 5, 183–191. doi: 10.1016/j.hpj.2019.06.001

Wang, Y., Ma, Z., Wang, X., Sun, Q., Dong, H., Wang, G., et al. (2019b). Effects of biochar on the growth of apple seedings, soil enzyme activities and fungal communities in replant disease soil. *Sci. Hortic.* 256:108641. doi: 10.1016/j.scienta.2019.108641

Xiang, L., Wang, M., Pan, F. B., Wang, G. S., Jiang, W. T., Wang, Y. F., et al. (2021). Transcriptome analysis *Malus domestica* 'm9t337' root molecular responses to *Fusarium solani* infection. *Physiol. Mol. Plant Pathol.* 113:101567. doi: 10.1016/j.pmpp.2020.101567

Xu, W. D., Zhu, Q., Liu, S., Dai, X. N., Zhang, B., Gao, C., et al. (2018). Calretinin participates in regulating steroidogenesis by PLC-Ca^{2+}-PKC pathway in leydig cells. *Sci. Rep.* 8:7403. doi: 10.1038/s41598-018-25427-3

Yang, Z. Q., Zhong, X. M., Fan, Y., Wang, H. C., Li, J. G., and Huang, X. M. (2015). Burst of reactive oxygen species in pedicel-mediated fruit abscission after carbohydrate supply was cut off in longan (*Dimocarpus longan*). *Front. Plant Sci.* 6:360. doi: 10.3389/fpls.2015.00360

Zhang, M. L., Huang, P. P., Ji, Y., Wang, S. W., Wang, S. S., Li, Z., et al. (2020). KUP9 maintains root meristem activity by regulating K^+ and auxin homeostasis in response to low K. *EMBO Rep.* 21:e50164. doi: 10.15252/embr.202050164

Zhang, T., Gao, Y., Han, M., and Yan, L. M. (2021). Changes in the physiological characteristics of *Panax ginseng* embryogenic calli and molecular mechanism of ginsenoside biosynthesis under cold stress. *Planta* 253:79. doi: 10.1007/s00425-020-03535-7

Zhang, X. H., Yang, D. Y., Zhang, E. H., Bai, C. C., and Wang, H. Z. (2013). Diurnal changes in photosynthesis and antioxidants of *Angelica sinensis* as influenced by cropping systems. *Photosynthetica* 51, 252–258. doi: 10.1007/s11099-013-0013-6

Zhang, Y. K., Han, X. J., Chen, X. L., Jin, H., and Cui, X. M. (2009). Exogenous nitricoxide on antioxidative system and ATPase activities from tomato seedlings under copper stress. *Sci. Hortic.* 123, 217–223. doi: 10.1016/j.scienta.2009.08.015

Zhao, D. Q., Li, T. T., Hao, Z. J., Cheng, M. L., and Tao, J. (2019). Exogenous trehalose confers high temperature stress tolerance to herbaceous peony by enhancing antioxidant systems, activating photosynthesis, and protecting cell structure. *Cell Stress Chaperones* 24, 247–257. doi: 10.1007/s12192-018-00961-1

Zhou, Z., Zhu, Y. M., Tian, Y., Yao, J. L., Bian, S. X., Zhang, H. T., et al. (2021). MdPR4, a pathogenesis-related protein in apple, is involved in chitin recognition and resistance response to apple replant disease pathogens. *J. Plant Physiol.* 260:153390. doi: 10.1016/j.jplph.2021.153390

Zhu, Y. M., and Saltzgiver, M. (2020). A systematic analysis of apple root resistance traits to *Pythium ultimum* infection and the underpinned molecular regulations of defense activation. *Hortic. Res.* 7:62. doi: 10.1038/s41438-020-0286-4

Zhu, Y. M., Shin, S., and Mazzola, M. (2016). Genotype responses of two apple rootstocks to infection by *Pythium ultimum* causing apple replant disease. *Can. J. Plant Pathol.* 38, 483–491. doi: 10.1080/07060661.2016.1260640

Zou, Q. J., Wang, S. T., Liang, K. J., Wang, Y. N., Hu, T. L., Han, Z. Q., et al. (2014). Suspected pathogenic *Fusarium* spp. isolated from apple orchard soils in Hebei Province (In Chinese). *Mycosystema* 33, 976–983. doi: 10.13346/j.mycosystema.120109

Author Disclaimer: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 December 2021
doi: 10.3389/fpls.2021.777871

Five Rootstocks for "Emperor" Mandarin Under Subtropical Climate in Southern Brazil

Maria Aparecida da Cruz[1,2,3], Carmen Silvia Vieira Janeiro Neves[2], Deived Uilian de Carvalho[2,3], Ronan Carlos Colombo[4], Jinhe Bai[1], Inês Fumiko Ubukata Yada[5], Rui Pereira Leite Junior[6] and Zuleide Hissano Tazima[3]*

[1] Horticultural Research Laboratory, ARS, United States Department of Agriculture (USDA), Fort Pierce, FL, United States, [2] Centro de Ciências Agrárias, Universidade Estadual de Londrina, Londrina, Brazil, [3] Área de Fitotecnia, Instituto de Desenvolvimento Rural do Paraná, Londrina, Brazil, [4] Centro de Ciências Agrárias, Universidade Federal Tecnológica do Paraná, Francisco Beltrão, Brazil, [5] Área de Biometria, Instituto de Desenvolvimento Rural do Paraná, Londrina, Brazil, [6] Área de Proteção de Plantas, Instituto de Desenvolvimento Rural do Paraná, Londrina, Brazil

OPEN ACCESS

Edited by:
Vittorino Novello, University of Turin, Italy

Reviewed by:
Davie Mayeso Kadyampakeni, University of Florida Gainesville, United States
Riccardo Lo Bianco, University of Palermo, Italy
Ebrahim Hadavi, Islamic Azad University of Karaj, Iran

***Correspondence:**
Maria Aparecida da Cruz
mary_ac18@hotmail.com

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

Received: *16 September 2021*
Accepted: *23 November 2021*
Published: *20 December 2021*

Citation:
Cruz MA, Neves CSVJ, Carvalho DU, Colombo RC, Bai J, Yada IFU, Leite Junior RP and Tazima ZH (2021) Five Rootstocks for "Emperor" Mandarin Under Subtropical Climate in Southern Brazil. Front. Plant Sci. 12:777871. doi: 10.3389/fpls.2021.777871

Rootstocks modulate several characteristics of citrus trees, including vegetative growth, fruit yield and quality, and resistance or tolerance to pests, diseases, soil drought, and salinity, among other factors. There is a shortage of scion and rootstock cultivars among the combinations planted in Brazil. "Ponkan" mandarin and "Murcott" tangor grafted on "Rangpur" lime comprise the majority of the commercial mandarin orchards in Brazil. This low genetic diversity of citrus orchards can favor pest and disease outbreaks. This study aimed to evaluate the agronomic performance, Huanglongbing (HLB) tolerance, and fruit quality of "Emperor" mandarin on five different rootstocks for nine cropping seasons under the subtropical soil-climate conditions of the North region of the state of Paraná, Brazil. The experimental design was a randomized block, with six replications, two trees per block, and five rootstocks, including "Rangpur" lime, "Cleopatra," and "Sunki" mandarins, "Swingle" citrumelo, and "Fepagro C-13" citrange. The evaluations included tree growth, yield performance, fruit quality, and HLB disease incidence. "Emperor" mandarin trees grafted on "Rangpur" lime and "Swingle" citrumelo had early fruiting and high yield efficiency. "Rangpur" lime also induced the lowest tree growth, but low fruit quality. Trees on "Swingle" citrumelo and "Fepagro C-13" citrange showed low scion and rootstock affinity and produced fruits with high total soluble solids (TSS), with a lower number of seeds for those from trees on "Fepagro C-13" citrange. "Cleopatra" and "Sunki" mandarins induced higher juice content, while fruits from trees on "Cleopatra" also had higher TSS/titratable acidity (TA) ratio. "Emperor" mandarin trees were susceptible to HLB regardless of the rootstocks. Overall, "Cleopatra" and "Sunki" mandarins, "Swingle" citrumelo, and "Fepagro C-13" are more suitable rootstocks for "Emperor" mandarin under Brazilian subtropical conditions than "Rangpur" lime.

Keywords: ***Citrus*** **spp., scion-rootstock combination, tree growth, fruit quality, yield performance, Huanglongbing**

INTRODUCTION

Mandarins are the second most important group of commercial citrus produced worldwide, next to oranges. In 2019, the total mandarin production globally was 35 million tons, with almost three-quarters produced in Asia [Food Agricultural Organization (FAO), 2019]. China is the largest mandarin producer, followed by Spain, Turkey, Morocco, Egypt, the United States, and Brazil [Food Agricultural Organization (FAO), 2019]. In 2020, over one million tons of mandarin fruits were produced in Brazil [Instituto Brasileiro de Geografia e Estatística (IBGE), 2020]. The Brazilian mandarin production is concentrated in the states of São Paulo, Minas Gerais, Paraná, and Rio Grande do Sul [Instituto Brasileiro de Geografia e Estatística (IBGE), 2020].

Despite the global importance of the Brazilian citrus industry, there is a shortage of citrus scion and rootstock cultivars. Among the cultivated mandarins and mandarin-like, "Ponkan" (*Citrus reticulata* Blanc.) and "Murcott" tangor [*C. reticulata* × *C. sinensis* (L.) Osb.], grafted mostly on "Rangpur" lime (*C. limonia* Osb.), are the most extensively used combinations in Brazil, representing 80% of the total mandarin acreage (Stuchi et al., 2008; Pacheco et al., 2017). Although preferred by the Brazilian consumers, the commercialization of "Ponkan" is restricted due to its short postharvest life (Carvalho S. A. et al., 2019). Under this scenario, the genetic diversification of citrus orchards, for both scion and rootstock cultivars, is important to prevent disease and pest outbreaks, and to extend the harvest season, as well as to improve the commercial performance of the citrus species under different edaphoclimatic conditions, producing fruits of high yield and quality (Emmanouilidoua and Kyriacoub, 2017; Carvalho L. M. et al., 2019; Alfaro et al., 2021).

Rootstocks determine several traits of the citrus trees, including vegetative growth, longevity, water and nutrient absorptions, yield performance, fruit quality, and tolerance or resistance to biotic and abiotic stresses (Castle, 1995, 2010; Castle et al., 2010; Pestana et al., 2011; Legua et al., 2014). The rootstocks included in this study were chosen according to their performance in previous studies in different citrus-growing areas using multiple scions. "Rangpur" lime has been the most used rootstock in Brazil for several decades, and with "Swingle" citrumelo [*C. paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.], are currently, the most important rootstock in the Brazilian citrus industry (Carvalho S. A. et al., 2019; Miranda et al., 2020). These rootstocks are between the 21 major world rootstocks in current use, along with "Cleopatra" (*C. reshni* Hort. ex Tan.) and "Sunki" (*C. sunki* Hort. ex Tan.) mandarins (Bowman and Joubert, 2020), also chosen to be evaluated in the present study. Although not extensively used, "Fepagro C-13" citrange [*C. sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.] was included in our study due to its higher horticultural performance reported in previous studies (Stenzel et al., 2003; Pompeu Junior and Blumer, 2014; Carvalho et al., 2021).

"Rangpur" lime is also used in other important citrus-growing areas, as China and India (Bowman and Joubert, 2020). This rootstock induces early fruiting and adequate production to the citrus trees (Pompeu Junior, 2005). In addition, "Rangpur" lime is compatible with most commercial citrus scions and is tolerant to the citrus Tristeza virus (CTV) (Pompeu Junior, 2005). Further, "Rangpur" lime is drought tolerant (Pedroso et al., 2014; Miranda et al., 2020). This last trait has gained more attention due to climate changes and the need for plants to adapt to a wide range of environmental conditions (Alfaro et al., 2021; Aparicio-Durán et al., 2021). However, the susceptibility of "Rangpur" lime to some diseases has raised concerns and need to search for new alternative rootstocks for the Brazilian citrus industry (Pompeu Junior and Blumer, 2014; Fadel et al., 2018; Carvalho L. M. et al., 2019; Carvalho S. A. et al., 2019; Carvalho et al., 2021).

"Swingle" citrumelo has become an alternative for rootstock diversification in several countries globally, including the United States, Spain, and Mexico (Castle et al., 2010; Cruz et al., 2019; Bowman and Joubert, 2020). Similar to "Rangpur" lime, "Swingle" citrumelo induces early fruiting and is resistant to CTV (Castle and Stover, 2000; Castle, 2010). Further, it is also resistant to nematodes, *Phytophthora nicotianae*, and citrus blight (Castle and Stover, 2000; Castle, 2010). "Cleopatra" mandarin has also been used in several citrus-growing areas. This rootstock induces excellent yields, vigorous growth, and shows tolerance to citrus blight, CTV, xyloporosis, and some abiotic stresses such as salinity, cold, and calcareous soils (Castle, 1987; Pompeu Junior, 2005). Similar to "Cleopatra," "Sunki" mandarin tolerates salinity, citrus blight, CTV, and xyloporosis and produces high-quality fruits and vigorous trees (Pompeu Junior, 2005). "Fepagro C-13" citrange is mostly used in Southern Brazil. This rootstock enhances the fruit yield and quality of the scion, besides being tolerant to some detrimental diseases and cold (Leite Junior, 1992; Stenzel et al., 2003; Pompeu Junior and Blumer, 2014; Carvalho et al., 2021).

As mentioned above, rootstocks are the key to facing challenges in the citrus industry. Currently, Huanglongbing (HLB) has been a major challenge to citrus production globally. The disease seriously affects citrus fruit quality and yield. Fruits from HLB-infected trees are usually reduced in size, sometimes asymmetric, greener, and have lower total soluble solids contents (TSS), higher titratable acidity (TA), and lower TSS/TA ratio (Dagulo et al., 2010; Dala-Paula et al., 2018, 2019). At present, there is no cure for HLB-infected trees (Bergamin Filho et al., 2016; Bassanezi et al., 2020). Recent studies revealed that some *Citrus* relatives seem to be more tolerant to HLB, by not showing typical HLB symptoms despite being infected (Albrecht and Bowman, 2012; Albrecht et al., 2016; Stover et al., 2016; Alves et al., 2021; Aparicio-Durán et al., 2021). However, no true resistance to the disease is known in the genus *Citrus* so far (Stover and McCollum, 2011; Albrecht and Bowman, 2012; Bergamin Filho et al., 2016).

The rootstock may perform differently when grafted with different scions. "Emperor" (*Citrus reticulata* Blanc.) is early to mid-season mandarin grown, mainly in Australia (Ladaniya, 2008). This mandarin is moderately resistant to citrus canker caused by the bacterium *Xanthomonas citri* subsp. *citri* (*Xcc*), a detrimental disease for the Brazilian citrus industry, with fruits of orange-colored, smooth skin, and seedy (Ladaniya, 2008; Leite Junior, 2015). Western Australia is the largest mandarin producer in Australia and has climatic conditions

similar to Southern Brazil, with maximum and minimum mean temperatures of 23 and 13°C, respectively [Bureau of Meteorology Western Australia (BOM), 2016]. "Emperor" may be a potential alternative for citrus scion diversification in Southern Brazil, as well as to other citrus-growing areas around the world, with a similar humid subtropical climate, such as Florida in the United States, East and South-Central China, and the coastal areas of Mexico. Accordingly, this study aimed to evaluate the influence of five rootstocks on the vegetative growth, yield performance, fruit quality, and HLB tolerance of "Emperor" mandarin under the humid subtropical climate of Southern Brazil.

MATERIALS AND METHODS

Experimental Location

The experiment was conducted at the Experimental Station of the Instituto de Desenvolvimento Rural do Paraná (IDR-Paraná) in Londrina, Paraná, Brazil (Latitude 23° 21′ 34″ S; Longitude 51° 09′ 53″ W; and altitude of 585 m). The soil is classified as Oxisol Typic Hapludox, a clay soil with a pH of 5.0 or higher and a base saturation (by NH_4OAc) of 35 % or less (U.S. Department of Agriculture, 1999), and the Köppen climate classification is Cfa (humid subtropical). The annual maximum and minimum mean temperatures are 27.3 and 16.1°C, respectively. The total annual rainfall is 1,639 mm (**Figure 1**) with a mean relative humidity of 70.5% [Instituto Agronômico do Paraná (IAPAR), 2018].

Plant Materials and Management

The experimental orchard was planted in December 2005, at a tree spacing of 7.0 m × 4.0 m between and within rows, respectively, with a planting density of 357 trees ha^{-1}. The orchard was not irrigated and weed control was performed periodically using an ecological rotary mower.

The experimental design was randomized blocks with five treatments (rootstocks), six blocks, and the data were collected from the two innermost trees of six trees per block. The rootstocks evaluated were "Rangpur" lime (*C. limonia* Osb.), "Cleopatra" mandarin (*C. reshni* Hort ex Tanaka), "Sunki" mandarin (*C. sunki* Hort ex Tanaka), "Swingle" citrumelo [*C. paradisi* Macf. cv. "Duncan" × *P. trifoliata* (L.) Raf.], and "Fepagro C-13" citrange [*C. sinensis* × *P. trifoliata* (L.) Raf]. Rootstock seeds and "Emperor" mandarin budwoods were obtained from the Citrus Active Germplasm Bank of the IDR-Paraná, in Londrina, Paraná, Brazil.

Trees were monitored periodically, and cultural practices were performed according to the recommendations for the state of Paraná, including preventative copper sprays to control citrus canker (*Xanthomonas citri* subsp. *citri*) and monthly insecticide applications to control the Asian citrus psyllid (*Diaphorina citri* Kuwayama) from 2014 up to 2016 [Instituto Agronômico do Paraná (IAPAR), 1992; Nunes et al., 2010]. The "Emperor" mandarin trees infected by the phloem-limited bacteria "*Candidatus* Liberibacter asiaticus," pathogen of (HLB), were eliminated.

Vegetative Growth

Vegetative growth was evaluated in the 2010 and 2016 seasons, after harvests. "Emperor" mandarin trees showed a broad-spread canopy with an oval shape, characteristic of the cultivar (Hodgson, 1967). The canopy volume was calculated based on tree height and canopy diameter, according to the equation proposed by Mendel (1956):

$$\boldsymbol{CV} = \frac{\mathbf{2}}{\mathbf{3}} \times \boldsymbol{\pi} \times \boldsymbol{CR^2} \times \boldsymbol{TH}$$

where **CV** = canopy volume (m^3); ***CR*** = canopy radius (m); and ***TH*** = tree height (m).

The trunk circumference was determined at 10 cm above (TDA) and below (TDB) the graft union and converted to diameter. Based on these measurements, the ratio between the trunk diameter below and above the graft union (TDB/TDA) was calculated. No pruning was performed at any stage of the tree growth.

Yield Performance

Fruit yield was determined annually in June, from 2008 to 2016 using a digital scale, and the results were expressed in fruit weight per tree. The cumulative yield was calculated by the sum of the annual yields. The yield efficiency of the trees was determined based on the ratio between fruit yield and canopy volume when the trees were 11 years old (2016). The alternate bearing index was determined according to Pearce and Dobersek-Urbanc (1967):

$$\mathbf{ABI} = \left(\frac{\mathbf{1}}{\boldsymbol{n-1}}\right) \times \left[\left(\frac{\boldsymbol{a_2 - a_1}}{\boldsymbol{a_2 + a_1}}\right) + \left(\frac{\boldsymbol{a_3 - a_2}}{\boldsymbol{a_3 + a_2}}\right) + \ldots + \left(\frac{\boldsymbol{a_{(n)} - a_{(n-1)}}}{\boldsymbol{a_{(n)} + a_{(n-1)}}}\right)\right]$$

where **ABI** = alternate bearing index; $\boldsymbol{n}$ = number of years; and $\boldsymbol{a_1}, \boldsymbol{a_2}, \ldots \boldsymbol{a_{(n)}}, \boldsymbol{a_{(n-1)}}$ = yields of the corresponding years.

Fruit Quality

The fruit quality was determined on 10 fruit samples per block. Samples were randomly collected at 1–2 m tree height in May for the seasons of 2012–2016, when the fruits reached maturity according to the international standards [Organization for Economic Co-operation and Development (OECD), 2010; Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP), 2011]. The fruit height and diameter were measured with a Vernier digital caliper (Mitutoyo, ABS, Kawasaki, Kanagawa, Japan) and used to determine the fruit shape (FH FD^{-1}). The fruits were weighted using a semi-analytic scale (total capacity of 15 kg) and classified according to the fresh citrus standards [Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP), 2011]. The juice was extracted in a Croydon extractor (Croydon, Duque de Caxias, Brazil). The juice content (JC) was determined based on the following equation and the results were expressed as a percentage:

$$\mathbf{JC} = \frac{\boldsymbol{JW}}{\boldsymbol{FW}} \times \mathbf{100}$$

where **JC** = juice content (%); ***JW*** = juice weight (g); and ***FW*** = fruit weight (g).

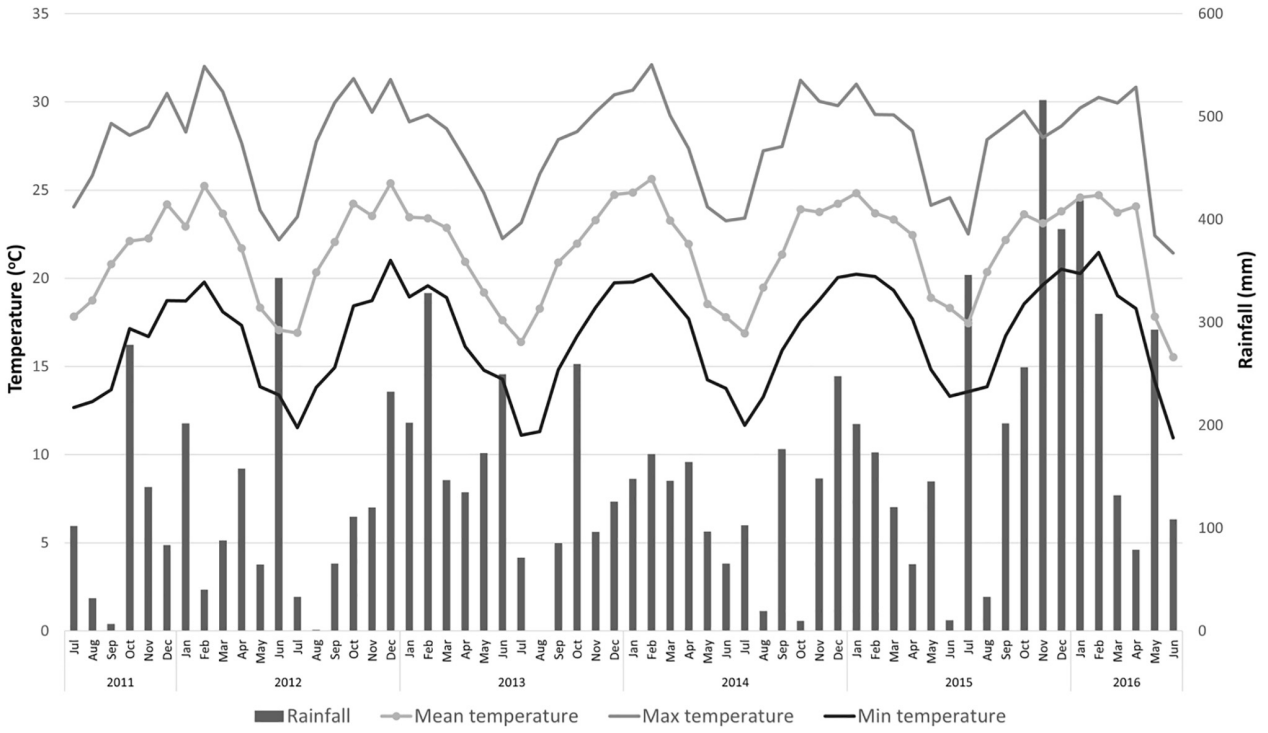

FIGURE 1 | Rainfall, and mean, maximum and minimum temperatures for 2011 through 2015 period in Londrina, Paraná, Brazil. [Source: Instituto Agronômico do Paraná (IAPAR), 2018].

The seeds were manually extracted from each fruit and counted to determine the number of seeds per fruit. The TSS was determined with a digital refractometer (Atago Co., Ltd., Tokyo, Japan) using 0.3 ml of undiluted juice. Values were expressed in percentage (≈ °Brix). The TA was determined by titrating 25 ml of juice with a standard solution of 0.1 N NaOH in an automatic titrator (TitroLine easy, Schott Instruments GmbH, Mainz, Rhineland-Palatinate, Germany). The acidity was expressed as the percentage of citric acid [Association of Official Analytical Chemists (AOAC) (2019)]. Then, the ratio between TSS and TA (TSS/TA) was used as the maturity indicator. The technological index (TI) or the amount of TSS per 40.8-kg box of fruits was determined according to the equation proposed by Di Giorgi et al. (1990).

$$\mathbf{TI} = \frac{\boldsymbol{TSS} \times \boldsymbol{JC} \times \mathbf{40.8}}{\mathbf{10000}}$$

where ***JC*** = juice content (%); and **40.8** = weight of the citrus industry standard box.

Incidence of Huanglongbing

The experimental orchard was periodically monitored by a trained crew. Trees were visually screened for the presence of typical HLB symptoms, such as asymmetric mottling and thickening of veins in mature leaves. The first symptoms of HLB in the orchard were detected in 2014. The presence of HLB-associated bacterium, "*Candidatus* Liberibacter asiaticus" (*C*Las), was confirmed by PCR. In 2014 and 2015, a 12-leaf sample per tree was collected from the two innermost trees of each block, and DNA extraction was performed according to the protocol described by Murray and Thompson (1980). *C*Las was detected by the conventional PCR technique, using the primers A2 and J5, specific to *C*Las (Hocquellet et al., 1999). The PCR test was performed using the protocol described by Bagio et al. (2016). The DNA bands were visualized under ultraviolet light (L-PIX EX, Loccus do Brasil Ltda, Cotia, Brazil). Once the presence of the *C*Las was confirmed, the HLB-infected trees were marked and eliminated after the harvest season every year, as the eradication of HLB-symptomatic trees is mandatory in Brazil. The rate of HLB infection was expressed as the percentage (%) of diseased trees.

Data Analyses

The experimental design was a randomized block, with five treatments (rootstocks) and six blocks. The data were tested for normal distribution and equal variance at $p \leq 0.05$. Square root transformations were used for all data that did not follow the normal distribution. All data were evaluated by ANOVA followed by the comparison of the means according to Tukey's test ($p \leq 0.05$). Fruit quality parameters were assessed in a randomized block design with a factorial arrangement, main factor 1: five rootstocks × main factor 2: five cropping seasons, the interaction between these factors was evaluated for each parameter. The statistical analyses were conducted using the *R* version 4.1.0 (The R Foundation for Statistical Computing, Vienna, Austria) and the ExpDes package (Husson et al., 2017).

RESULTS

"Emperor" mandarin trees grafted on all evaluated rootstocks did not show any significant differences ($p \leq 0.05$) in height, canopy diameter, and volume at the beginning of the trial (**Table 1**). However, in 2016, the trees grafted on "Rangpur" lime had smaller tree height and canopy volume than those on the other rootstocks, though did not differ from trees on "Fepagro C-13" for canopy volume (**Table 1**).

The trees on "Swingle" citrumelo had the largest TDB in 2010 and 2016 (**Table 1**). Trees on "Fepagro C-13" citrange showed the smallest TDA in both evaluated years (**Table 1**). While trees on "Sunki" mandarin showed the largest TDA on both evaluations (**Table 1**). Furthermore, the TDB/TDA was significantly higher for the trees on "Swingle" and "Fepagro C-13," compared with those on the other rootstocks in both seasons (**Table 1**).

The fruit yield of the "Emperor" mandarin trees grafted on the different rootstocks had a wide fluctuation across the experimental period (**Table 2**). In the first harvest, trees on "Rangpur" lime and "Swingle" citrumelo had higher yields than the other rootstocks, indicating earliness in fruit production (**Table 2**). The trees on "Swingle" citrumelo had the highest yields per tree in almost all seasons, except for 2010 (**Table 2**). The cumulative yield of the "Emperor" mandarin trees were not affected by the rootstock (**Table 2**). The yield efficiency was higher for trees grafted on "Rangpur" lime and "Swingle" citrumelo than those on other rootstocks (**Table 2**). "Fepagro C-13" citrange induced the lowest yield efficiency to "Emperor" mandarin (**Table 2**). The alternate bearing index of the "Emperor" mandarin trees was not affected by the rootstock, and the values ranged from 0.32 up to 0.50 (**Table 2**).

Significant interactions ($p \leq 0.001$) were observed between harvest season and rootstock for all fruit quality parameters, except TSS (**Tables 3**, **4**). Fruits from the trees on "Rangpur" lime showed an alternate in height, diameter, and weight across the evaluated period (**Table 3**). Fruits were smaller and lighter in the 2012, 2014, and 2016 seasons than those in the 2013 and 2015 seasons (**Table 3**). No differences in fruit height, diameter, shape, and weight were observed between the treatments for the 2016 season. In general, the "Emperor" fruits were smaller, lighter, and nearly round in shape for all scion-rootstock combinations in 2016, compared with fruits from the other seasons (**Table 3**).

"Emperor" fruits from all scion-rootstock combinations had height:diameter ratios above 0.86 in most of the evaluated seasons, indicating a nearly round shape (**Table 3**). In 2012 and 2014, fruits from trees grafted on all rootstocks, except on "Sunki" mandarin in both years and on "Fepagro C-13" citrange in 2012, were below 0.83, indicating a moderately oblate shape (**Table 3**). Fruits from the trees on "Rangpur" lime scored the lowest fruit weights in almost all cropping seasons (**Table 3**).

Fruits from trees on "Fepagro C-13" citrange showed a lower number of seeds in almost all evaluated seasons (**Table 3**). "Sunki" and "Cleopatra" mandarins induced the production of fruits with similar juice content across the evaluated period (**Table 3**). These fruits were among those with higher juice content in most of the evaluated years, except in 2012 (**Table 3**). On the other hand, fruits from the trees on "Rangpur" lime had low juice content in most of the seasons (**Table 3**). Fruits produced in the 2014 and 2016 seasons had lower juice content than those from the other seasons (**Table 3**).

As main effects, harvest season and rootstock were highly significant ($p \leq 0.001$) for TSS over the five seasons, but no significant interaction between these factors was observed (**Table 4**). The TSS content was significantly higher in fruits produced by trees on "Fepagro C-13" and "Swingle" than those from trees on "Rangpur" (**Table 4**). Regarding the TSS per season, the values were relatively lower in 2013, 2015, and 2016 (**Table 4**). The TA was low for fruits from trees on all rootstocks in the first three seasons and increased in 2015 and 2016 (**Table 4**). "Swingle" citrumelo induced higher TA to "Emperor" mandarin fruits than the other rootstocks evaluated, being among those with the highest TA over the evaluated period (**Table 4**). On the other hand, "Rangpur" lime induced low TA to "Emperor" fruits in almost all seasons, except for 2014 (**Table 4**). The TSS/TA ratio was lower for fruits from the trees on all rootstocks in 2015 and 2016 compared to the TSS/TA ratio of fruits from the other seasons. "Emperor" mandarin fruits from trees on "Cleopatra" were among those with the highest TSS/TA ratio over the evaluated period (**Table 4**). Overall, the TSS and TSS/TA ratio were lower, and the TA was higher for fruits from trees on all scion-rootstock combinations in 2015 and 2016 compared with the other seasons (**Table 4**).

The TI varied through the seasons and was not influenced by the rootstocks in 2014 and 2015 (**Table 4**). However, there was a positive interaction between rootstock and cropping season ($p \leq 0.001$). Fruits from all scion-rootstock combinations had lower TI in 2014 and 2016 than the fruits from the other seasons (**Table 4**).

The tree infection rate for HLB was 10% in 2014 and 30% in 2015, comprising a total of 40% of diseased trees (**Table 5**). The entire grove was eliminated in 2016, due to the high incidence of the disease. There was no statistical difference in the incidence of the disease between the evaluated rootstocks (**Table 5**).

DISCUSSION

The vegetative growth of the scion is directly affected by the rootstock, related to the genotype and its relationships (Auler et al., 2008). The vegetative growth of the "Emperor" mandarin trees observed in our study was similar to those of "Okitsu" satsumas and "Ponkan" mandarins, which also showed smaller tree size, i.e., height and canopy volume, when grafted on "Rangpur" lime and "Fepagro C-13" citrange, compared with those on the other rootstocks (Stenzel et al., 2003; Tazima et al., 2013). Similarly, the smallest growth pattern of the citrus trees grafted on "Rangpur" lime was reported for "Sunburst" and "Oneco" mandarins (Mourão Filho et al., 2007; Gonzatto et al., 2011), supporting the low vigor conferred by this rootstock to different scions.

The use of rootstocks that induce lower tree height and canopy volume allows the increase in plant density by area, which is a tendency in modern citrus production (Auler et al., 2008; Stover et al., 2008; Pompeu Junior and Blumer, 2009). High-density

TABLE 1 | Vegetative growth of "Emperor" mandarin trees grafted on five different rootstocks for the 2010 and 2016 cropping seasons. Londrina, Paraná, Brazil.

Rootstock	Tree height (m)		Canopy diameter (m)		Canopy volume (m^3)		TDB[2] (cm)		TDA[2] (cm)		TDB/TDA[3]	
	2010	2016	2010	2016	2010	2016	2010	2016	2010	2016	2010	2016
"Rangpur" lime	2.2 a[1]	2.7 b	2.7 a	3.9 a	8.3 a	21.9 b	32.2 b	47.6 c	24.6 ab	35.0 bc	1.3 b	1.4 c
"Cleopatra" mandarin	2.1 a	3.1 a	2.6 a	4.2 a	7.9 a	29.4 a	31.9 b	52.4 c	23.2 bc	37.1 ab	1.4 b	1.4 c
"Sunki" mandarin	2.4 a	3.1 a	2.8 a	4.2 a	9.7 a	29.0 a	33.4 b	55.0 bc	25.5 a	40.3 a	1.3 b	1.4 c
"Swingle" citrumelo	2.3 a	3.0 a	2.7 a	4.3 a	9.0 a	29.0 a	40.1 a	69.0 a	22.6 cd	33.6 cd	1.8 a	2.1 a
"Fepagro C-13" citrange	2.3 a	2.9 a	2.6 a	4.0 a	8.0 a	24.7 ab	35.5 ab	60.0 b	21.3 d	31.4 d	1.7 a	1.9 b
CV (%)[4]	6.50	4.70	7.44	5.28	16.56	11.68	7.86	7.22	4.29	5.12	7.97	4.85
Block	0.111^{ns}	0.047^{*}	0.494^{ns}	0.165^{ns}	0.224^{ns}	0.060^{ns}	0.270^{ns}	0.062^{ns}	0.266^{ns}	0.027^{ns}	0.554^{ns}	0.008^{**}
Rootstock	0.097^{ns}	0.000^{***}	0.582^{ns}	0.064^{ns}	0.263^{ns}	0.002^{**}	0.000^{***}	0.000^{***}	0.000^{***}	0.000^{***}	0.000^{***}	0.000^{***}

[1]Means followed by the same letter in the column did not differ according to Tukey's test ($p \leq 0.05$). [2]Trunk diameters were based on trunk circumference measured 10 cm below (TDB) and above (TDA) the graft union. [3]TDA/TDA, the ratio between scion and rootstock trunk diameter. [4]Coefficient of variation (CV). p-value: ^{ns}, non-significant, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

TABLE 2 | Annual and cumulative yields, relative yield, yield efficiency, and yield alternate bearing index of "Emperor" mandarin trees grafted on five different rootstocks through nine consecutive cropping seasons (2008–2016) in Londrina, Paraná, Brazil.

Season	Yield (kg $tree^{-1}$)					CV[2] (%)	*p*-value	
	"Rangpur" lime	"Cleopatra" mandarin	"Sunki" mandarin	"Swingle" citrumelo	"Fepagro C-13" citrange		Block	Rootstock
2008	39.7 a[1]	23.8 b	18.8 c	31.1 a	17.2 c	22.99	0.012^{*}	0.000^{***}
2009	38.8 a	48.1 a	60.6 a	61.8 a	44.8 a	36.03	0.082^{ns}	0.166^{ns}
2010	63.3 a	60.2 a	25.3 b	30.3 b	49.1 ab	36.49	0.697^{ns}	0.002^{**}
2011	46.6 b	86.1 a	93.6 a	79.2 a	75.9 a	18.61	0.492^{ns}	0.000^{***}
2012	131.5 a	91.3 ab	80.0 b	118.7 ab	127.9 a	21.56	0.641^{ns}	0.003^{**}
2013	21.5 b	65.1 a	83.0 a	47.9 ab	69.3 a	35.54	0.378^{ns}	0.000^{***}
2014	24.4 c	34.9 bc	35.5 bc	51.4 ab	54.9 a	25.05	0.031^{*}	0.000^{***}
2015	29.6 b	79.3 a	69.0 ab	47.9 ab	27.0 b	56.67	0.729^{ns}	0.016^{*}
2016	78.4 ab	64.8 b	69.8 b	100.4 a	33.1 c	23.95	0.521^{ns}	0.000^{***}
Cumulative yield	473.8 a	553.6 a	535.5 a	568.7 a	499.3 a	14.00	0.978^{ns}	0.192^{ns}
Yield efficiency (kg m^{-3})[3]	3.7 a	2.2 b	2.4 b	3.5 a	1.4 c	26.16	0.375^{ns}	0.000^{***}
Alternate bearing index	0.39 a	0.32 a	0.50 a	0.40 a	0.40 a	14.61	0.798^{ns}	0.196^{ns}

[1]Means followed by the same letter in the row did not differ statistically according to Tukey's test ($p \leq 0.05$). [2]Coefficient of variation (CV). [3]The calculated yield efficiencies correspond only to the 2016 season. p-value: ^{ns}, non-significant, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

orchards maximize fruit quality and yield, decrease harvest costs, and facilitate crop management (Stover et al., 2008; Pompeu Junior and Blumer, 2009; Stuchi et al., 2012). In addition, higher densities orchards may improve profitability for farmers in HLB-endemic areas, under the removal of HLB-symptomatic trees (Moreira et al., 2019). In our study, no pruning was performed at any stage of the tree growth. However, in commercial orchards, scion-rootstock combinations with small vegetative growth may require less frequent pruning. This can result in less frequent emission of new shoots, which may contribute to a decrease in the attack of the Asian citrus psyllid (Stuchi et al., 2012).

The largest trunk diameter below the graft union reported for trees on "Swingle" citrumelo is a well-known characteristic conferred by this rootstock to several citrus species. Trees on "Swingle" citrumelo grow vigorously and show a trunk overgrowth near to the grafting union (Castle et al., 1988). Similar overgrowth has been observed for "Okitsu" satsuma mandarin and "Navelina," "Jaffa," "Cadenera," and "Salustiana" sweet orange trees grafted on "Swingle" citrumelo (Tazima et al., 2013; Bacar et al., 2017; Domingues et al., 2018; Cruz et al., 2019; Carvalho et al., 2021).

The ratio between the trunk diameter below and above the graft union (TDB/TDA) may be an indication of scion and rootstock compatibility (Tazima et al., 2013), where indices close to one have been usually considered as the good affinity between them (Fadel et al., 2018). The highest TDB/TDA ratios of the "Emperor" trees were observed for those on "Swingle" citrumelo and "Fepagro C-13" citrange (**Table 1**). Similar TDB/TDA ratios were reported for other mandarins and sweet oranges grafted on the same rootstocks, such as "Marisol" clementine (Bassal, 2009), "Navelina" sweet orange (Cruz et al., 2019), and "Okitsu" satsuma mandarin (Tazima et al., 2013, 2014). However, the differences in trunk diameters between scion and rootstock may not be related to graft-incompatibility in some cases and may not

TABLE 3 | Physical quality of "Emperor" mandarin fruits from trees grafted on five different rootstocks, in Londrina, Paraná, Brazil.

Rootstock	Fruit height (FH, mm)					Fruit diameter (FD, mm)				
	2012	2013	2014	2015	2016	2012	2013	2014	2015	2016
"Rangpur" lime	45.0 cD[1]	59.1 aB	45.1 cD	65.2 aA	52.7 aC	58.7 dC	63.5 abB	55.6 cC	69.0 aA	58.5 aC
"Cleopatra" mandarin	53.3 bAB	53.0 bcAB	50.2 bcB	55.2 cA	52.6 aAB	66.3 abA	59.9 bBC	58.2 bcC	64.2 abAB	58.7 aC
"Sunki" mandarin	61.1 aA	52.0 cB	63.2 aA	53.4 cB	50.9 aB	70.7 aA	60.3 bB	70.4 aA	61.0 bB	58.2 aB
"Swingle" citrumelo	53.2 bB	58.6 abA	49.9 bcB	58.6 bcA	52.1 aB	65.2 bcA	63.5 aA	60.9 bAB	64.9 abA	58.4 aB
"Fepagro C-13" citrange	49.8 bcC	57.4 acA	56.6 bAB	61.5 abA	51.6 aBC	60.9 cdBC	63.0 abAB	66.5 aA	65.7 abA	57.8 aC
CV (%)			6.14					2.40		
Block			0.097^{ns}					0.202^{ns}		
Rootstocks			0.000***					0.000***		
Year			0.000***					0.000***		
Rootstock × Year			0.000***					0.000***		
Rootstock	**Fruit shape (FH/FD)**					**Fruit weight (g)**				
	2012	2013	2014	2015	2016	2012	2013	2014	2015	2016
"Rangpur" lime	0.77 cC	0.93 aA	0.81 cB	0.95 aA	0.90 aA	88.8 dC	120.5 abB	72.3 bC	161.8 aA	95.6 aC
"Cleopatra" mandarin	0.80 bcC	0.88 bcA	0.83 bcBC	0.86 bAB	0.89 aA	134.8 abA	104.4 bBC	150.1 aA	127.9 bcAB	97.0 aC
"Sunki" mandarin	0.86 aB	0.86 cB	0.91 aA	0.87 bAB	0.89 aAB	152.5 aA	102.2 bB	156.2 aA	112.5 cB	96.6 aB
"Swingle" citrumelo	0.82 bB	0.89 acA	0.82 bcB	0.90 abA	0.89 aA	121.3 bcA	130.9 aA	95.0 bB	130.7 bcA	95.6 aB
"Fepagro C-13" citrange	0.82 bC	0.91 abA	0.86 bB	0.94 aA	0.89 aAB	100.5 cdBC	119.0 abAB	131.9 aA	137.7 abA	93.3 aC
CV (%)			1.59					13.13		
Block			0.046*					0.069^{ns}		
Rootstocks			0.000***					0.000***		
Year			0.000***					0.000***		
Rootstock × Year			0.000***					0.000***		
Rootstock	**Number of seeds**					**Juice content (%)**				
	2012	2013	2014	2015	2016	2012	2013	2014	2015	2016
"Rangpur" lime	26.3 aA	18.8 aB	25.1 aA	18.7 abB	20.2 bB	36.5 bB	42.7 aA	31.3 abC	37.4 bB	31.9 bC
"Cleopatra" mandarin	27.6 aA	19.7aB	21.7 abB	22.4 aB	21.9 abB	36.5 bBC	42.0 abA	34.4 aC	39.2 abAB	33.8 abC
"Sunki" mandarin	25.5 aA	20.1 aB	19.8 bB	22.0 aAB	19.6 bB	33.4 bB	41.1 abA	32.3 abB	41.8 aA	35.5 aB
"Swingle" citrumelo	23.5 aA	18.1 aB	21.9 abAB	21.1 aAB	22.0 abAB	35.3 bB	38.8 bcA	30.5 bC	40.7 abA	34.0 abB
"Fepagro C-13" citrange	24.0 aA	12.3 bC	19.6 bB	15.8 bBC	25.0 aA	40.7 aA	36.7 cB	31.9 abC	40.6 abA	34.5 abBC
CV (%)[2]			12.88					5.89		
Block			0.768^{ns}					0.185^{ns}		
Rootstocks			0.000***					0.060^{ns}		
Year			0.000***					0.000***		
Rootstock × Year			0.000***					0.000***		

*[1]Means followed by the same lowercase letters in the column or uppercase letters in the row did not differ statistically according to Tukey's test. [2]Coefficient of variation (CV). p-value: ^{ns}, non-significant, *$p \leq 0.05$; ***$p \leq 0.001$.*

always influence the horticultural performance of the scion (Fadel et al., 2018). Although the larger differences noticed in our study between scion and rootstock trunk diameters were for the trees on "Swingle" citrumelo and "Fepagro C-13" citrange, these trees did not show any symptoms of incompatibility or decay in the first eleven years after planting.

"Emperor" mandarin had early fruit production when the trees were grafted on "Rangpur" lime and "Swingle" citrumelo (**Table 2**). Trees grafted on these two rootstocks usually bear fruits at an early stage (Castle and Stover, 2000; Bowman and Joubert, 2020). This finding is in agreement with those reported for other citrus cultivars as "Okitsu" satsumas and "Oneco" mandarins, and the "Jaffa," "Navelina," and "Salustiana" sweet oranges (Gonzatto et al., 2011; Tazima et al., 2013; Bacar et al., 2017; Cruz et al., 2019; Carvalho et al., 2021). Rootstocks that induce early fruiting to the citrus trees are preferable, especially under the current HLB situation. HLB infection results in a short productive life of the citrus trees, reducing the economic life of the groves to less than 10 years due to the severity of the symptoms and the fast disease spread (Stover et al., 2008; Bové, 2012; Albrigo et al., 2019). Therefore, it is necessary that production reaches high levels early and

TABLE 4 | Chemical quality of "Emperor" mandarin fruits of trees grafted on five different rootstocks, in Londrina, Paraná, Brazil.

Rootstock	Total soluble solids (TSS,%)	Titratable acidity (TA, %)				
	mean	2012	2013	2014	2015	2016
"Rangpur" lime	9.8 b[1]	0.91 bcB	0.92 bB	1.00 aB	1.12 bA	1.12 cA
"Cleopatra" mandarin	10.1 ab	0.78 dD	0.99 abC	0.87 cCD	1.30 aA	1.18 cB
"Sunki" mandarin	10.1 ab	0.85 cdC	1.08 aB	0.90 bC	1.38 aA	1.39 aA
"Swingle" citrumelo	10.4 a	0.99 abB	1.00 abB	1.06 aB	1.36 aA	1.30 abA
"Fepagro C-13" citrange	10.3 a	1.06 aBC	0.94 bC	0.97 abC	1.18 bAB	1.19 bcA
2012	11.2 A	–	–	–	–	–
2013	9.8 C	–	–	–	–	–
2014	10.6 B	–	–	–	–	–
2015	9.5 C	–	–	–	–	–
2016	9.7 C	–	–	–	–	–
CV (%)	5.13			7.06		
Block	0.656^{ns}			0.349^{ns}		
Rootstocks	0.000***			0.000***		
Year	0.000***			0.000***		
Rootstock × Year	0.141^{ns}			0.000***		

Rootstock	Ratio (TSS/TA)					Technological index TI (kg TSS box^{-1})				
	2012	2013	2014	2015	2016	2012	2013	2014	2015	2016
"Rangpur" lime	12.0 bA	10.2 abB	9.9 bB	8.0 aC	8.6 aC	1.64 bA	1.64 abA	1.33 aB	1.56 aA	1.19 bB
"Cleopatra" mandarin	13.9 aA	10.1 abC	11.9 aB	7.5 abD	8.3 aD	1.62 bAB	1.70 aA	1.38 aC	1.51 aBC	1.37 abC
"Sunki" mandarin	13.3 aA	9.2 bC	11.8 aB	6.8 bD	6.8 bD	1.53 bAB	1.67 abA	1.39 aB	1.63 aA	1.35 abB
"Swingle" citrumelo	11.5 bA	9.8 abB	10.3 bB	7.2 abC	7.8 abC	1.63 bA	1.56 abAB	1.36 aB	1.61 aA	1.44 aB
"Fepagro C-13" citrange	11.0 bA	10.6 aA	11.6 aA	8.1 aB	7.9 abB	1.90 aA	1.48 bBC	1.45 aBC	1.56 aB	1.31 abC
CV (%)[2]			7.3					8.18		
Block			0.369^{ns}					0.146^{ns}		
Rootstocks			0.000***					0.279^{ns}		
Year			0.000***					0.000***		
Rootstock × Year			0.000***					0.000***		

*[1]Means followed by the same lowercase letters in the column or uppercase letters in the row did not differ statistically according to Tukey's test. [2]Coefficient of variation (CV). p-value: ^{ns}, non-significant, ***$p \leq 0.001$.*

maintains it for as long as possible during the orchard life (Stover et al., 2008).

The highest yields induced by "Swingle" citrumelo to the "Emperor" mandarin trees over the cropping seasons have also been reported previously for "Okitsu" satsuma mandarins, and "Navelina" and "Valencia" sweet oranges (Pompeu Junior and Blumer, 2011; Tazima et al., 2013; Cruz et al., 2019). However, the rootstocks did not influence the "Emperor" mandarin cumulative yield, though trees on "Swingle," "Cleopatra," and "Sunki" had yields 20, 17, and 13% higher than the trees on "Rangpur" lime, respectively (**Table 2**). The yield efficiency was higher for trees grafted on "Rangpur" lime and "Swingle" citrumelo than those on other rootstocks (**Table 2**). The yield efficiency is based on fruit production and canopy volume, "Rangpur" lime induced lower canopy volume to "Emperor" mandarin, which contributed to its high yield efficiency (**Table 1**). The use of rootstocks, which induce lower tree vegetative growth and high yield efficiency, enables the increase in plant densities per area, increasing fruit yield and facilitating harvest and crop management (Stover et al., 2008; Pompeu Junior and Blumer, 2009; Cruz et al., 2019).

We found no effect of the rootstocks on the alternate bearing of the "Emperor" mandarin trees (**Table 2**). Alternate bearing is common in mandarins and is characterized by irregular fruit production over the years (Siqueira and Salomão, 2016). The alternate bearing index ranges from 0 up to 1, where values closer to 0 indicate lower yield alternation (Tazima et al., 2014). Therefore, "Emperor" mandarin trees show low alternate bearing indices under subtropical conditions, ranging from 0.32 up to 0.50, regardless of the rootstock they were grafted on (**Table 2**). Similar results, with no effect of the rootstock, were reported for "Okitsu" satsuma, "Flagallo," "Sunburst," and "Span Americana" mandarins (Mourão Filho et al., 2007; Silva et al., 2013; Tazima et al., 2014). A high alternate bearing index usually results in small fruits with low quality in years of overproduction (Siqueira and Salomão, 2016). Therefore, the low alternate bearing revealed in our study for "Emperor" mandarin on multiple rootstocks can favor the production of fruits with better size and quality over the years.

Mandarins are produced primarily for the fresh fruit market (Albrigo et al., 2019). Although fresh citrus fruits must meet

TABLE 5 | Incidence of Huanglongbing (HLB) disease on "Emperor" mandarin trees grafted on five different rootstocks in Londrina, Paraná, Brazil.

Rootstock	HLB-affected trees (%)		
	2014	2015	Total
"Rangpur" lime	0[1]	33	33
"Cleopatra" mandarin	17	33	50
"Sunki" mandarin	8	17	25
"Swingle" citrumelo	25	17	42
"Fepagro C-13" citrange	0	50	50
CV (%)[2]	21.19	23.97	23.47
[3]Block	0.78^{ns}	0.91^{ns}	0.97^{ns}
[3]Rootstock	0.18^{ns}	0.34^{ns}	0.71^{ns}

[1]Means followed by the same letter in the column did not differ according to Tukey's test ($p \leq 0.05$). [2]Coefficient of variation (CV). [3]p-value: ^{ns}, non-significant.

internal quality standards, the external appearance and fruit size are very important for consumer acceptance (Albrigo et al., 2019; Tarancón et al., 2021). Consumers' preferences for fresh citrus fruits include seedless fruits with optimal size and shape and easily removable peel (Spreen et al., 2020). Generally, medium to large fruits provides higher returns to the growers (Hussain et al., 2013). The minimal mandarin fruit diameter accepted by the international fresh citrus market is 45 mm [Organization for Economic Co-operation and Development (OECD), 2010]. "Emperor" mandarin fruits of the trees on all evaluated rootstocks had larger diameters than the minimum standard (**Table 3**).

Fruit size is influenced by several factors, such as cultivar, rootstock, crop load, climate, and cultural practices (Albrigo et al., 2019). The variation on fruit size and weight observed in this study for fruits produced by trees grafted on "Rangpur" lime, may be related to the annual fruit load (**Tables 2**, **3**). In 2012 and 2016, "Emperor" mandarin trees on "Rangpur" lime reached higher fruit yields, with fruits being smaller and lighter than those in the other seasons (**Tables 2**, **3**). Crop load has a significant impact on citrus fruit size, with the final fruit size being inversely related to the number of fruits that reach maturity (Goldschmidt and Monselise, 1977; Guardiola and Lazaro, 1987; Agustí et al., 1999). This phenomenon is attributed to the competition between the developing organs for photosynthates and mineral elements (Albrigo et al., 2019). The high number of developing organs leads to strong competition for photosynthates and mineral elements and, consequently, to smaller final fruit sizes.

The fruit shape of mandarins may range from oblate to round (Goldenberg et al., 2018). A height:diameter ratio closer to 1 indicates a round shape, while a ratio around 0.65 indicates an oblate shape (Goldenberg et al., 2014, 2018). In this study, the "Emperor" mandarin fruits had shapes ranging from moderated oblate (0.77–0.83) to nearly round (0.86–0.95), depending on the rootstock and crop season (**Table 3**). Only fruits from the trees on "Sunki" mandarin presented a nearly round shape in all evaluations (**Table 3**).

The "Emperor" mandarin fruit weight was similar or heavier than those reported for this cultivar in a previous study, 90–100 g (Ladaniya, 2008). Except for those fruits from trees on "Rangpur" lime in the 2012 and 2014 seasons, that weighted less than 90 g (**Table 3**). Low fruit weight on fruits from trees grafted on "Rangpur" lime was also reported for the "Folha Murcha" sweet orange (Stenzel et al., 2005). In 2016, fruits from trees on all rootstocks had weights lower than 100 g (**Table 3**). Higher water supply during fruit development leads to an increase in fruit size and weight (Romero et al., 2006; Albrigo et al., 2019). However, the fruits of the 2016 season were smaller and lighter than those of the other seasons (**Table 3**), even with a high rainfall volume during fruit development (**Figure 1**). This was probably due to an increase in HLB infection in the grove at that season (**Table 5**). It is well known that HLB infection decreases the size and weight of citrus fruits (Dala-Paula et al., 2018, 2019).

"Emperor" mandarin fruits are seedy (Ladaniya, 2008). In this study, the number of seeds per fruit ranged from 12 up to 28 seeds per fruit, for those from trees on "Fepagro C-13" and "Cleopatra" respectively (**Table 3**). The number of seeds per fruit found in our study is similar or even fewer than those reported in distinct mandarins and hybrids, including "Cravo," "Nules," and "Murcott" tangor (Pio et al., 2005; Pacheco et al., 2017). Fruits from the trees on "Fepagro C-13" citrange showed the lowest number of seeds per fruit on most evaluations (**Table 3**). This is a desirable characteristic, as seedless fruits or fruits with a low number of seeds are preferable to the consumer (Albrigo et al., 2019; Spreen et al., 2020). Although, studies on consumer preference in the United States, suggested that sweetness, shape, acidity, and flavor are more important factors to the purchase decision than the number of seeds (House et al., 2011; Baldwin et al., 2014).

Juice content is another important quality parameter for the commercialization and consumption of citrus fruits. Citrus containing lower juice content than the commercial standards [Organization for Economic Co-operation and Development (OECD), 2010; Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP), 2011] are depreciated at the fresh and industrial markets as the fruit became tasteless with low levels of organic acids and soluble solids, reducing the saleable weight of the fruit that causes economic loss (Jones and Cree, 1965; Ladaniya, 2008). Fruits from trees grafted on the mandarin rootstocks were among those with the highest juice content in most evaluations, while fruits from trees on "Rangpur" lime exhibited the lowest juice content for this period (**Table 3**). Regardless of the rootstock, the juice contents of "Emperor" mandarin fruits were above the minimal standard of the international fresh citrus market, which is 33% [Organization for Economic Co-operation and Development (OECD), 2010], in almost all crop seasons. However, fruits solely produced by trees on "Cleopatra" reached this requirement in the 2014 season (**Table 3**), as this parameter is dependent on several factors that include soil-climate conditions, nutritional balance, field management, and water relations (**Figure 1**; Castle, 2010; Albrigo et al., 2019). Previous work has confirmed this trend conferred by "Cleopatra" for "Lane Late" sweet orange (Emmanouilidoua and Kyriacoub, 2017). Regarding the Brazilian fresh citrus market, the minimal marketable juice content for mandarin and hybrid fruits is 35% [Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP), 2011]. Based on this threshold,

"Emperor" mandarin trees grafted on most evaluated rootstocks produced fruits that reached this baseline in the 2012, 2013, and 2015 seasons (**Table 3**). However, in the 2014 and 2016 seasons, almost all "Emperor"-rootstock combinations produced fruits with low juice content, below this standard (**Table 3**), which evidences the need for better management adoption for "Emperor" mandarin, as fruit thinning and irrigation system that in terms regulate fruit quality.

Although the external appearance of the mandarin fruits is very important, changes in the chemical internal quality of the fruit determine the maturity level (Albrigo et al., 2019). Citrus are classified as non-climacteric fruits and must be harvested when the internal maturity has been achieved, as no further relevant maturation changes will occur in these fruits after harvest (Lado et al., 2014; Albrigo et al., 2019). As mandarin fruit matures, the TSS content increases and the TA levels decrease, in which TSS becomes nearly constant or increases slightly at the late stage of fruit development (Ladaniya, 2008; Albrigo et al., 2019). In general, the balance between sugars and organic acids in juice is the main indicator of mandarin maturity (Ladaniya, 2008; Lado et al., 2014; Goldenberg et al., 2018).

Based on our results, "Emperor" mandarin juice peaked the highest TSS content in the 2012 and 2014 seasons (**Table 4**). This fact may be related to the climatic conditions, as trees were not irrigated and relied on natural rainfall. Lower rainfall volumes were recorded during these seasons, prior to the harvest time, which may have regulated the fruit quality, particularly in 2012 (**Figure 1**). According to previous studies, there is an increase in TSS accumulation in fruits of "Satsuma" mandarin trees under water stress, because of the increase in the osmotic potential and sucrose hydrolysis (Yakushiji et al., 1998; Barry et al., 2004). The authors support that this effect is independent of the fruit size and juice content, and is not caused by passive dehydration. However, the water stress can also cause dehydration in the fruit and consequently higher accumulation of TSS (Stenzel et al., 2006), which is supported by the low juice content reported in the 2012 and 2014 seasons (**Table 3**).

The rootstock also had a large effect on TSS accumulation. Fruits from trees on "Swingle" citrumelo and "Fepagro C-13" citrange showed higher TSS content than those on "Rangpur" lime (**Table 4**). This may be caused by differences in tree water status influenced by the rootstock (Barry et al., 2004). Previous studies have reported high TSS content in fruits of "Okitsu" satsuma mandarin on "Swingle" citrumelo and "Fepagro C-13" citrange rootstocks (Tazima et al., 2014). Whereas, low TSS scores were found in fruits of "Michal," "Fallglo," and "Sunburst" mandarin trees grafted on "Rangpur" lime, supporting our findings in the present study (Mourão Filho et al., 2007; Brugnara et al., 2009). Despite the differences, "Emperor" fruits produced by trees on all tested rootstocks reached TSS above 9%, which surpasses the minimal standard established for the fresh citrus market [Organization for Economic Co-operation and Development (OECD), 2010; Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP), 2011].

The TA of citrus juices is also an important factor in overall juice quality and in determining the time of harvest (Harding et al., 1940). According to Pereira et al. (2006), the citric acid level in mature mandarin fruits must range between 0.5 and 1.0%. The TA levels recorded for "Emperor" mandarin fruits were close to those obtained for "Clementine" (0.70–1.20%) and "Okitsu" satsuma (0.88–1.03%) mandarins (Georgiou, 2002; Tazima et al., 2014). "Swingle" citrumelo induced the highest TA level to "Emperor" mandarin fruits, while "Rangpur" lime imparted the lowest TA means in most seasons (**Table 4**). Similar results were reported for "Michal" (Brugnara et al., 2009), "Okitsu" satsuma (Cantuarias-Avilés et al., 2010), and "Oneco" mandarins (Gonzatto et al., 2011). Some studies suggest that the rootstock can influence the fruit maturity stage, by delaying or advancing it, allowing an extension of the commercial season for the canopy cultivar (Stenzel et al., 2006; Morales et al., 2020). The lowest acidity loss exerted by "Swingle" citrumelo associated with the high TSS may prolong the commercial period of the "Emperor" mandarin, by still imparting good TSS/AT at the end of the season, while fruits from the trees on "Rangpur" lime may be tasteless and flat by that time (Morales et al., 2020). Although some consumers do not prefer acidic fruits, the lack of acidity turns the fruit tasteless and flat, unsuitable for fresh consumption (Ladaniya, 2008). A fluctuation in TA level was observed over the evaluated period. "Emperor" mandarin juice showed low acid content in the first three seasons and increased significantly in the last two seasons (2015 and 2016), being higher than 1.12 for all scion-rootstock combinations (**Table 4**). This was probably related to the HLB infection in the orchard in those seasons (**Table 5**). The citrus fruits produced by HLB-infected trees usually have disease low TSS and TSS/ratio and high TA (Dagulo et al., 2010; Dala-Paula et al., 2018, 2019).

The acceptability of TSS/TA ratios for the commercialization of mandarin fruits varies according to the target market and usually fluctuates from 7 up to 9:1 (Albrigo et al., 2019). Fruits produced on all scion-rootstock combinations showed TSS/TA ratio higher than 8.5 in the first years of evaluation (**Table 4**), which meets the standard requirements of the Brazilian fresh citrus market [Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP), 2011]. On the other hand, only "Emperor" mandarin fruits from trees on "Rangpur" lime reached the minimal standard in the 2016 season (**Table 4**). The lowest TSS content and the highest TA recorded in the last 2 years of evaluation have contributed to the decrease of the index (**Table 4**). Although the effect on the content of sugars and acids depends on the rootstock/scion interaction, some rootstocks have the same effect on different cultivars (Albrigo et al., 2019). The high ratio observed for "Emperor" mandarin fruits from trees on "Cleopatra" in this study (**Table 4**) is consistent with those reported for "Marisol" mandarin (Bassal, 2009) and "Valencia" sweet orange (Bowman et al., 2016).

The TI is an important qualitative parameter for the processing industry, in which higher TI values mean fewer boxes of fruits needed to produce one ton of frozen concentrate orange juice (FCOJ) at 65°Brix, as this index indicates the amount of TSS in a standard citrus box of 40.8 kg (Di Giorgi et al., 1990). Although mandarins are primarily commercialized in the fresh fruit market, due to their deep color and quality, the citrus industry may use mandarin juice to blend with orange or other fruit juices to improve their color and odor/aroma or to sell the juice as single strength (Pérez-López et al., 2006; Albrigo et al., 2019).

In our study, the TIs were low in the 2014 and 2016 seasons (**Table 4**). It may be related to the low juice content reported in these seasons since TI is based on TSS and juice content (Di Giorgi et al., 1990). The technological indices observed for "Emperor" mandarin over the evaluated period were slightly lower than the ones reported by Tazima et al. (2014) for "Okitsu" satsuma mandarin grafted in the same rootstocks.

The natural occurrence of Huanglongbing (HLB) in our experimental orchard has shown that all tested rootstocks combined with "Emperor" mandarin are susceptible to the disease (**Table 5**). Although there was no statistical difference, "Emperor" trees grafted on "Cleopatra" and "Fepagro C-13" rootstocks had a higher incidence of HLB compared with all other combinations (**Table 5**). These results corroborate previous reports, in which trees on "Cleopatra" mandarin were the most affected by HLB (Lopes and Frare, 2008; Albrecht and Bowman, 2012). The effect of the disease on fruit quality was evidenced in this study in the last two evaluated years when the infection rate in the grove was higher (**Tables 3–5**). In general, "Emperor" mandarin juice scored lower for TSS and TSS/TA ratio, but higher for TA (**Table 4**). The external qualitative parameters, including fruit size and weight, decreased significantly in 2016 compared to the previous seasons (**Table 3**) which have compromised the marketable value of the fruits. These results are important for the citrus industry as there still have a lack of studies regarding the HLB effect on mandarin fruit quality; however, our findings are in agreement with those reported for sweet oranges, in which the effects of the disease were plenty studied (Dagulo et al., 2010; Liao and Burns, 2012; Massenti et al., 2016; Baldwin et al., 2018; Dala-Paula et al., 2018).

In general, the trees grafted on "Rangpur" lime had the lowest vegetative growth, high yield efficiency, and started fruiting early. However, this scion-rootstock combination produced fruits with lower fruit quality compared with the other tested scion-rootstock combinations. These fruits exhibited low fruit weight, juice content, and TSS. Trees on "Swingle" citrumelo and "Fepagro C-13" citrange showed the lowest scion-rootstock affinity, however, no clear signs of incompatibility were observed in the trees. These rootstocks also induced higher TSS to "Emperor" mandarin fruits. Fruits from trees on "Fepagro C-13" citrange also showed few number of seeds; however, this rootstock induced the lowest yield efficiency. Trees grafted on "Swingle" citrumelo started to bear fruits early and showed high yields over the nine cropping seasons with high yield efficiency. "Cleopatra" and "Sunki" mandarins induced higher juice content for "Emperor" mandarin across the evaluated period. Fruits produced by trees on "Cleopatra" exhibited a higher TSS/TA ratio.

CONCLUSION

Rootstocks significantly influenced the tree vegetative growth, fruit yield, and quality of "Emperor" mandarins. Based on our findings, "Cleopatra" and "Sunki" mandarins, "Swingle" citrumelo, and "Fepagro C-13" citrange are more suitable rootstocks for "Emperor" mandarins under the Brazilian subtropical conditions than "Rangpur" lime. Despite inducing low tree size, early fruiting, and high yield efficiency, "Rangpur" lime induced lower fruit quality compared with the other rootstock options. The rootstock choice should be made depending on their specific characteristics and the prevalent interest of the region/market. "Swingle" citrumelo induces early fruiting, high fruit yield and yield efficiency, and good fruit quality with high TSS content. "Fepagro C-13" imparts good fruit quality with a low number of seeds per fruit and high TSS, but low yield efficiency to "Emperor" mandarin. While "Cleopatra" and "Sunki" mandarins induce high juice content. "Cleopatra" also imparts a high TSS/TA ratio to "Emperor" mandarin fruits.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MC: data collection, formal data analysis, and writing—original draft. CN: supervision, writing—review and editing, and resources. DC: formal data analysis and writing—review and editing. RC: formal data analysis and investigation. JB: writing—review and editing. IY: investigation. RL: conceptualization, writing—review and editing, and resources. ZT: conceptualization, supervision, writing—review and editing, funding acquisition, resources, and investigation. All authors approved the submission.

FUNDING

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (Grant No. 88887.634596/2017-01) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq.

REFERENCES

Agustí, M., Almela, V., and Juan, M. (1999). Comportamiento agronómico de la mandarina 'Loretina'. *Levante Agríc.* 347, 134–152.

Albrecht, U., and Bowman, K. D. (2012). Tolerance of trifoliate citrus rootstock hybrids to *Candidatus Liberibacter* asiaticus. *Sci. Hortic.* 147, 71–80. doi: 10.1016/j.scienta.2012.08.036

Albrecht, U., Fiehn, O., and Bowman, K. D. (2016). Metabolic variations in different citrus rootstock cultivars associated with different responses to huanglongbing. *Plant Physiol. Biochem.* 107, 33–44. doi: 10.1016/j.plaphy.2016.05.030

Albrigo, L. G., Stelinski, L. L., and Timmer, L. (2019). *Citrus*. Boston, MA: CAB International. doi: 10.1079/9781845938154.0000

Alfaro, J. M., Bermejo, A., Navarro, P., Quiñones, A., and Salvador, A. (2021). Effect of rootstock on *Citrus* fruit quality: a review. *Food Rev. Int.* 37, 1–19. doi: 10.1080/87559129.2021.1978093

Alves, M. N., Lopes, S. A., Raiol-Junior, L. L., Wulff, N. A., Girardi, E. A., Ollitrault, P., et al. (2021). Resistance to '*Candidatus* Liberibacter asiaticus,' the huanglongbing associated bacterium, in sexually and/or graft-compatible *Citrus* relatives. *Front. Plant Sci.* 11:617664. doi: 10.3389/fpls.2020.617664

Association of Official Analytical Chemists (AOAC) (2019). *Official Methods of Analysis of the AOAC International*. Arlington, TX: AOAC International.

Aparicio-Durán, L., Gmitter, F. G. Jr., Arjona-López, J. M., Calero-Velázquez, R., Hervalejo, Á, and Arenas-Arenas, F. J. (2021). Water-stress influences on three new promising HLB-tolerant *Citrus* rootstocks. *Horticulturae* 7:336. doi: 10.3390/horticulturae7100336

Auler, P. A. M., Fiori-Tutida, A. C. G., and Tazima, Z. H. (2008). Behavior of 'Valencia' orange tree on six rootstocks in the Northwest of Parana state. *Rev. Bras. Frutic.* 30, 229–234. doi: 10.1590/S0100-29452008000100042

Bacar, E. L. C., Neves, C. S. V. J., Leite Junior, R. P., Yada, I. F. U., and Tazima, Z. H. (2017). 'Jaffa' sweet orange plants grafted onto five rootstocks. *Rev. Bras. Frutic.* 39:e200. doi: 10.1590/0100-29452017200

Bagio, T. Z., Canteri, M. G., Barreto, T. P., and Leite Junior, R. P. (2016). Activation of systemic acquired resistance in *Citrus* to control huanglongbing disease. *Semina* 37, 1757–1766. doi: 10.5433/1679-0359.2016v37n4p1757

Baldwin, E. A., Bai, J., Plotto, A., and Ritenour, M. A. (2014). *Citrus* fruit quality assessment; producer and consumer perspectives. *Stewart Postharvest Rev.* 10, 1–7.

Baldwin, E. A., Plotto, A., Bai, J., Manthey, J., Zhao, W., Raithore, S., et al. (2018). Effect of abscission zone formation on orange (*Citrus sinensis*) fruit/juice quality for trees affected by huanglongbing (HLB). *J. Agric. Food Chem.* 66, 2877–2890. doi: 10.1021/acs.jafc.7b05635

Barry, G. H., Castle, W. S., and Davies, F. S. (2004). Rootstocks and plant water relations affect sugar accumulation of *Citrus* fruit *via* osmotic adjustment. *J. Am. Soc. Hortic. Sci.* 129, 881–889. doi: 10.21273/JASHS.129.6.0881

Bassal, M. A. (2009). Growth, yield and fruit quality of 'Marisol' clementine grown on four rootstocks in Egypt. *Sci. Hortic.* 119, 132–137. doi: 10.1016/j.scienta.2008.07.020

Bassanezi, R. B., Lopes, S. A., Miranda, M. P., Wulff, N. A., Volpe, H. X. L., and Ayres, A. J. (2020). Overview of citrus huanglongbing spread and management strategies in Brazil. *Trop. Plant Pathol.* 45, 251–264. doi: 10.1007/s40858-020-00343-y

Bergamin Filho, A., Nagata, A., Bassanezi, R. B., Belasque Junior, J., Amorim, L., Macedo, M. A., et al. (2016). The importance of primary inoculum and area-wide disease management to crop thealth and fodd security. *Food Sec.* 8, 221–238. doi: 10.1007/s12571-015-0544-8

Bureau of Meteorology Western Australia (BOM) (2016). *Climate Statistics for Australian Locations*. West Perth, WA: Bureau of Meteorology Western Australia.

Bové, J. M. (2012). Huanglongbing and the future of citrus in São Paulo state, Brazil. *J. Gen. Plant Pathol.* 94, 465–467. doi: 10.4454/JPP.V94I3.001

Bowman, K. D., McCollum, G., and Albrecht, U. (2016). Performance of 'Valencia' orange (*Citrus sinensis* [L.] Osbeck) on 17 rootstocks in a trial severely affected by huanglongbing. *Sci. Hortic.* 201, 355–361. doi: 10.1016/j.scienta.2016.01.019

Bowman, K., and Joubert, J. (2020). "Citrus rootstocks," in *The Genus Citrus*, eds M. Talon, M. Caruso, and F. G. Gmitter Jr. (Cambridge: Woodhead Publishing), 105–127. doi: 10.1016/B978-0-12-812163-4.00006-1

Brugnara, E. C., Schwarz, S. F., Koller, O. C., Bender, R. J., Weiler, R. L., Gonzatto, M. P., et al. (2009). Porta-enxertos para a tangerineira 'Michal' no Rio Grande do Sul. *Ciênc. Rural* 39, 1374–1379.

Cantuarias-Avilés, T., Mourão Filho, F. A. A., Stuchi, E. S., Silva, S. R., and Espinoza-Núñez, E. (2010). Tree performance and fruit yield and quality of 'Okitsu' Satsuma mandarin grafted on 12 rootstocks. *Sci. Hortic.* 123, 318–322. doi: 10.1016/j.scienta.2009.09.020

Carvalho, D. U., Neves, C. S. V. J., Cruz, M. A., Colombo, R. C., Yada, I. F. U., Leite-Junior, R. P., et al. (2021). Performance of 'Salustiana' sweet orange on different rootstocks under Brazilian subtropical conditions. *Sci. Hortic.* 287:110226. doi: 10.1016/j.scienta.2021.110226

Carvalho, L. M., Carvalho, H. W., Barros, I., Martins, C. R., Soares Filho, W. D. S., Girardi, E. A., et al. (2019). New scion-rootstock combinations for diversification of sweet orange orchards in tropical hard setting soils. *Sci. Hortic.* 243, 169–176. doi: 10.1016/j.scienta.2018.07.032

Carvalho, S. A., Girardi, E. A., Mourão Filho, F. D. A. A., Ferrarezi, R. S., and Coletta Filho, H. D. (2019). Advances in citrus propagation in Brazil. *Rev. Bras. Frutic.* 41:e422. doi: 10.1590/0100-29452019422

Castle, B., and Stover, E. (2000). Rootstock reflections: swingle citrumelo update. *Citrus Ind.* 81, 18–20.

Castle, W. S. (1987). "Citrus rootstocks," in *Rootstocks for Fruit Crops*, eds R. C. Rom and R. F. Carlson (Hoboken, NJ: John Wiley and Sons), 361–399.

Castle, W. S. (1995). Rootstock as a fruit quality factor in citrus and deciduous tree crops. *N. Z. J. Crop. Hortic.* 23, 383–394. doi: 10.1080/01140671.1995.9513914

Castle, W. S. (2010). A career perspective on *Citrus* rootstocks, their development, and commercialization. *HortScience* 45, 11–15. doi: 10.21273/HORTSCI.45.1.11

Castle, W. S., Baldwin, J. C., and Muraro, R. P. (2010). Performance of 'Valencia' sweet orange trees on 12 rootstocks at two locations and an economic interpretation as a basis for rootstock selection. *HortScience* 45, 523–533. doi: 10.21273/HORTSCI.45.4.523

Castle, W. S., Wutscher, H. K., Yontsey, C. O., and Pelosi, R. R. (1988). Citrumelos as rootstocks for Florida *Citrus*. *Proc. Fla. State Hortic. Soc.* 101, 28–33.

Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP) (2011). *Normas de Classificação de Citros de Mesa*. São Paulo: CEAGESP.

Cruz, M. A., Neves, C. S. V. J., Carvalho, D. U., Colombo, R. C., Leite, R. P., and Tazima, Z. H. (2019). 'Navelina' sweet orange trees on five rootstocks in Northern Parana state, Brazil. *Rev. Bras. Frutic.* 41:e006. doi: 10.1590/0100-29452019006

Dagulo, L., Danyluk, M. D., Spann, T. M., Valim, M. F., Goodrich-Schneider, R., Sims, C., et al. (2010). Chemical characterization of orange juice from trees infected with citrus greening (huanglongbing). *J. Food Sci.* 75, 199–207. doi: 10.1111/j.1750-3841.2009.01495

Dala-Paula, B. M., Plotto, A. B., Manthey, J. A., Baldwin, E. A., Ferrarezi, R. S., and Gloria, M. B. A. (2019). Effect of huanglongbing or greening disease on orange juice quality, a review. *Front. Plant Sci.* 9:1976. doi: 10.3389/fpls.2018.01976

Dala-Paula, B. M., Raithore, S., Manthey, J. A., Baldwin, E. A., Bai, J., Zhao, W., et al. (2018). Active taste compounds in juice from oranges symptomatic for huanglongbing (HLB) *Citrus* greening disease. *Food Sci. Technol.* 91, 518–525. doi: 10.1016/j.lwt.2018.01.083

Di Giorgi, F., Ide, B. Y., Dib, K., Marchi, R. J., Triboni, H. R., and Wagner, R. L. (1990). Contribuição ao estudo do comportamento de algumas variedades de citros e suas implicações agroindustriais. *Citrus Res. Tech.* 11, 567–612.

Domingues, A. R., Neves, C. S. V. J., Yada, I. F. U., Leite Junior, R. P., and Tazima, Z. H. (2018). Performance of 'Cadenera' orange trees grafted on five rootstocks. *Rev. Bras. Frutic.* 40:e764. doi: 10.1590/0100-29452018764

Emmanouilidoua, M. G., and Kyriacoub, M. (2017). Rootstock-modulated yield performance, fruit maturation and phytochemical quality of 'Lane Late' and 'Delta' sweet orange. *Sci. Hortic.* 225, 112–121. doi: 10.1016/j.scienta.2017.06.056

Fadel, A. L., Mourão Filho, F. A. A., Stuchi, E. S., and Ramos, Y. C. (2018). Production of 'Valência' sweet orange on 41 rootstocks in the north of the state of São Paulo, Brazil. *Pesq. Agropec. Bras.* 6, 774–778. doi: 10.1590/S0100-204X2018000600015

Food Agricultural Organization (FAO) (2019). *FAOSTAT: Production Crops*. Available online at: http://www.fao.org/faostat/en/#data/QC/visualize (accessed May 17, 2021)

Georgiou, A. V. (2002). Evaluation of rootstocks for 'Clementine' mandarin in Cyprus. *Sci. Hortic.* 93, 29–38. doi: 10.1016/S0304-4238(01)00311-9

Goldenberg, L., Yaniv, Y., Kaplunov, T., Doron-Faigenboim, A., Porat, R., and Carmi, N. (2014). Genetic diversity among mandarins in fruit-quality traits. *J. Agric. Food Chem.* 62, 4938–4946. doi: 10.1021/jf5002414

Goldenberg, L., Yaniv, Y., Porat, R., and Carmi, N. (2018). Mandarin fruit quality: a review. *J. Sci. Food Agric.* 98, 18–26. doi: 10.1002/jsfa.8495

Goldschmidt, E. E., and Monselise, S. P. (1977). Physiological assumptions toward the development of a *Citrus* fruiting model. *Proc. Int. Soc. Citric.* 2, 668–672.

Gonzatto, M. P., Kovaleski, A. P., Brugnara, E. C., Weiler, R. L., Sartori, I. A., de Lima, J. G., et al. (2011). Performance of 'Oneco' mandarin on six rootstocks in South Brazil. *Pesq. Agropec. Bras.* 46, 406–411. doi: 10.1590/S0100-204X2011000400010

Guardiola, J. L., and Lazaro, E. (1987). Factors limiting productivity in *Citrus*. A physiological approach. *Proc. Int. Soc. Citric.* 1, 381–394.

Harding, P. L., Winston, J. R., and Fisher, D. F. (1940). *Seasonal Changes in Florida Oranges.* (Technical Bulletin, No. 753). Washington, DC: USDA.

Hocquellet, A., Toorawa, P., Bove, J. M., and Garnier, M. (1999). Detection and identification of the two *Candidatus* liberibacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the b operon. *Mol. Cell. Probes* 13, 373–379. doi: 10.1006/mcpr.1999.0263

Hodgson, R. W. (1967). "Horticultural varieties of *Citrus*," in *The Citrus Industry*, eds W. Reuther, H. J. Webber, and L. D. Batchelor (Los Angeles, CA: University of California), 431–591.

House, L. A., Gao, Z., Spreen, T. H., Gmitter, F. G., Valim, M. F., Plotto, A., et al. (2011). Consumer preference for mandarins: implications of a sensory analysis. *Agribusiness* 27, 450–464. doi: 10.1002/agr.20275

Hussain, S., Curk, F., Anjum, M. A., Pailly, O., and Tison, G. (2013). Performance evaluation of common clementine on various citrus rootstocks. *Sci. Hortic.* 150, 278–282. doi: 10.1016/j.scienta.2012.11.010

Husson, F., Lê, S., and Pages, J. (2017). *Exploratory Multivariate Analysis by Example Using R*. Boca Raton, FL: Chapman and Hall.

Instituto Agronômico do Paraná (IAPAR) (1992). *A Citricultura no Paraná*. Londrina: IAPAR. (IAPAR Circular, 72).

Instituto Agronômico do Paraná (IAPAR) (2018). *Médias Históricas em Estações do IAPAR*. Available online at: www.iapar.br/arquivos/Image/monitoramento/Medias_Historicas/Londrina.htm (accessed August 17, 2019)

Instituto Brasileiro de Geografia e Estatística (IBGE) (2020). *Levantamento Sistemático da Produção Agrícola*. Available online at: https://sidra.ibge.gov.br/Tabela/1613 (accessed October 05, 2021).

Jones, W. W., and Cree, C. B. (1965). Environment factors related to fruiting of Washington navel orange over a 38-year period. *Proc. Am. Soc. Hortic. Sci.* 86, 267–271.

Ladaniya, M. S. (2008). *Citrus Fruit: Biology, Technology and Evaluation*. Goa: ICAR Research Complex for Goa.

Lado, J., Zacarías, L., and Rodrigo, M. J. (2014). Maturity indicators and citrus quality. *Stewart Postharvest Rev.* 10, 1–6.

Legua, P., Forner, J. B., Hernández, F. C. A., and Forner- Giner, M. A. (2014). Total phenolics, organic acids, sugars and antioxidant activity of mandarin (*Citrus clementina* Hort. Ex Tan.): variation from rootstock. *Sci. Hortic.* 174, 60–64. doi: 10.1016/j.scienta.2014.05.004

Leite Junior, R. P. (1992). "Cultivares de copa e porta-enxertos," in *A Citricultura no Paraná*, ed IAPAR (Londrina: IAPAR), 93–116.

Leite Junior, R. P. (2015). *Cancro Cítrico: Prevenção e Controle*. Available online at: www.adapar.pr.gov.br/arquivos/File/GSV/CFO/II_Semana_Integrada_Cursos_CFO_2015/Apresentacoes/Cancro_Citrico.pdf (accessed August 17, 2020)

Liao, H. K., and Burns, J. (2012). Gene expression in *Citrus sinensis* fruit tissues harvested from huanglongbing-infected trees: comparison with girdled fruit. *J. Exp. Bot.* 63, 3307–3319. doi: 10.1093/jxb/ers070

Lopes, S. A., and Frare, G. F. (2008). Graft transmission and cultivar reaction of citrus to '*Candidatus* liberibacter americanus'. *Plant Dis.* 92, 21–24. doi: 10.1094/PDIS-92-1-0021

Massenti, R., Lo Bianco, R., Sandhu, A. K., Gu, L., and Sims, C. (2016). Huanglongbing modifies quality components and flavonoid content of 'Valencia' oranges. *J. Sci. Food Agric.* 96, 73–78. doi: 10.1002/jsfa

Mendel, K. (1956). Rootstock-scion relationships in Shamouti trees on light soil. *Ktavim* 6, 35–60.

Miranda, M. T., Da Silva, S. F., Silveira, N. M., Pereira, L., Machado, E. C., and Ribeiro, R. V. (2020). Root osmotic adjustment and stomatal control of leaf gas exchange are dependent on *Citrus* rootstocks under water deficit. *J. Plant Growth Regul.* 40, 11–19. doi: 10.1007/s00344-020-10069-5

Morales, J., Bermejo, A., Navarro, P., and Salvador, A. (2020). Rootstock efect on physico-chemical and nutritional quality of mandarin 'Clemenules' during the harvest season. *Agronomy* 10:1350. doi: 10.3390/agronomy10091350

Moreira, A. S., Stuchi, E. S., Silva, P. R., Bassanezi, R. B., Girardi, E. A., and Laranjeira, F. F. (2019). Could tree density play a role in managing *Citrus* huanglongbing epidemics? *Trop. Plant Pathol.* 44, 268–274. doi: 10.1007/s40858-019-00284-1

Mourão Filho, F. A. A., Espinoza-Núñez, E., Stuchi, E. S., and Ortega, E. M. M. (2007). Plant growth, yield, and fruit quality of 'Fallglo' and 'Sunburst' mandarins on four rootstocks. *Sci. Hortic.* 114, 45–49. doi: 10.1016/j.scienta.2007.05.007

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8, 4321–4325. doi: 10.1093/nar/8.19.4321

Nunes, W. N. C., de Souza, E. B., Leite Junior, R. P., Salvador, C. A., Rinaldi, D. A., Croce Filho, J., et al. (2010). Plano de ação para o controle de huanglongbing no estado do Paraná, Brasil. *Citrus Res. Tech.* 31, 169–177. doi: 10.5935/2236-3122.20100017

Organization for Economic Co-operation and Development (OECD) (2010). *Citrus Fruits, International Standards for Fruit and Vegetables*. Paris: OECD Publishing.

Pacheco, C. A., Azevedo, F. A., Barros, V. N. P., Cristofani-Yaly, M., and Verruma-Bernardi, M. R. (2017). Fremont – IAC 543: tangerine withpotential for the brazilian market. *Rev. Bras. Frutic.* 39:e436. doi: 10.1590/0100-29452017436

Pearce, S. C., and Dobersek-Urbanc, S. (1967). The measurement of irregularity in growth and cropping. *J. Hortic. Sci.* 42, 295–305. doi: 10.1080/00221589.1967.11514216

Pedroso, F. K. J. V., Prudente, D. A., Bueno, A. C. R., Machado, E. C., and Ribeiro, R. V. (2014). Drought tolerance in citrus trees is enhanced by rootstock-dependent changes in root growth and carbohydrate availability. *Environ. Exp. Bot.* 101, 26–35. doi: 10.1016/j.envexpbot.2013.12.024

Pereira, M. E. C., Cantillano, F. F., Gutierez, A. S. D., and Almeida, G. V. B. (2006). *Post Harvest Procedures in Integrated Production of Citrus*. Cruz das Almas: Embrapa Mandioca e Fruticultura. (Documentos, 156).

Pérez-López, A. J., Saura, D., Lorente, J., and Carbonell-Barrachina, A. A. (2006). Limonene, linalool, α-terpineol, and terpinen-4-ol as quality control parameters in mandarin juice processing. *Eur. Food Res. Technol.* 222, 281–285. doi: 10.1007/s00217-005-0055-5

Pestana, M., Correia, P. J., David, D., Abadía, A., Abadía, J., and Varennes, A. (2011). Response of five citrus rootstocks to iron deficiency. *J. Plant Nutr. Soil Sci.* 174, 837–846. doi: 10.1002/jpln.201000341

Pio, R. M., Figueiredo, J. O., Stuchi, E. S., and Cardoso, S. A. B. (2005). "Variedades copas," in *Citros*, eds D. M. Mattos Junior, J. D. Negri, R. M. Pio, and J. Pompeu Junior (Campinas: Instituto Agronômico e Fapesp), 39–57.

Pompeu Junior, J. (2005). "Porta-enxertos," in *Citros*, eds D. M. Mattos Junior, J. D. Negri, R. M. Pio, and J. Pompeu Junior (Campinas: Instituto Agronômico e Fapesp), 63–94.

Pompeu Junior, J., and Blumer, S. (2009). Trifoliate hybrids as rootstocks for sweet orange 'Valência'. *Pesq. Agropec. Bras.* 44, 701–705. doi: 10.1590/S0100-204X2009000700008

Pompeu Junior, J., and Blumer, S. (2011). Citrandarins e outros híbridos de trifoliata como porta-enxertos para laranjeira Valência. *Citrus RT* 32, 133–138.

Pompeu Junior, J., and Blumer, S. (2014). Híbridos de trifoliata como porta-enxertos para laranjeira Pêra. *Pesq. Agropec. Trop.* 44, 9–14. doi: 10.1590/S1983-40632014000100007

Romero, P., Navarro, J. M., Pérez-Pérez, J., García-Sánchez, F., Gómez-Gómez, A., Porras, I., et al. (2006). Deficit irrigation and rootstock: their effects on water relations, vegetative development, yield, fruit quality and mineral nutrition of Clemenules mandarin. *Tree Physiol.* 26, 1537–1548. doi: 10.1093/treephys/26.12.1537

Silva, S. R., Stuchi, E. S., Girardi, E. A., Cantuarias-Avilés, T., and Bassan, M. M. (2013). Performance of 'Span Americana' mandarin on different rootstocks. *Rev. Bras. Frutic.* 35, 1052–1058. doi: 10.1590/S0100-29452013000400016

Siqueira, D. L., and Salomão, L. C. C. (2016). *Citros: Do Plantio à Colheita*. Viçosa: UFV.

Spreen, T. H., Gao, Z., Fernandes Junior, W., and Zansler, M. L. (2020). "Global economics and marketing of citrus products," in *The Genus Citrus*, eds M. Talon, M. Caruso, and F. G. Gmitter Jr. (Cambridge: Woodhead Publishing), 471–493. doi: 10.1016/B978-0-12-812163-4.00023-1

Stenzel, N. M. C., Neves, C. S. V. J., Gomes, J. C., and Medina, C. C. (2003). Performance of 'Ponkan' mandarin on seven rootstocks in Southern Brazil. *HortScience* 38, 176–178. doi: 10.21273/HORTSCI.38.2.176

Stenzel, N. M. C., Neves, C. S. V. J., Gonzalez, M. G. N., Scholz, M. B. S., and Gomes, J. C. (2005). Vegetative growth, yield and quality fruits of the 'Folha Murcha' orange on six rootstocks in North Parana, Brazil. *Ciênc. Rural* 35, 1281–1286.

Stenzel, N. M. C., Neves, C. S. V. J., Marur, C. J., Scholz, M. B. S., and Gomes, J. C. (2006). Maturation curves and degree-days accumulation for fruits of 'Folha murcha' orange trees. *Sci. Agric.* 63, 219–225.

Stover, E., and McCollum, G. (2011). Incidence and severity of Huanglongbing and *Candidatus* liberibacter asiaticus titer among field-infected citrus cultivars. *HortScience* 46, 1344–1348. doi: 10.21273/HORTSCI.46.10.1344

Stover, E., Castle, W. S., and Spyke, P. (2008). The citrus grove of the future and its implications for huanglongbing management. *Proc. Fla. State Hortic. Soc.* 121, 155–159.

Stover, E., Inch, S., Richardson, M. L., and Hall, D. G. (2016). Conventional citrus of some scion/rootstock combinations show field tolerance under high huanglongbing disease pressure. *HortScience* 51, 127–132.

Stuchi, E. S., Espinoza-Núñez, E., Mourão Filho, F. A. A., and Ortega, E. M. M. (2008). Vigor, produtividade e qualidade de frutos de quatro tangerineiras e híbridos sobre quatro porta-enxertos. *Rev. Bras. Frutic.* 30, 741–747. doi: 10.1590/S0100-29452008000300030

Stuchi, E. S., Girardi, E. A., Sempionato, O. R., Reiff, E. T., Silva, S. R., and Parolin, L. G. (2012). *Trifoliata 'Flying Dragon': Porta-Enxerto Para Plantios Adensados e Irrigados de Laranjeiras Doces de Alta Produtividade e Sustentabilidade*. Cruz das Almas: Embrapa Mandioca e Fruticultura. (Documentos, 152).

Tarancón, P., Tárrega, A., González, M., and Besada, C. (2021). External quality of mandarins: influence of fruit appearance characteristics on consumer choice. *Foods* 10:2188. doi: 10.3390/foods10092188

Tazima, Z. H., Neves, C. S. V. J., Yada, I. F. U., and Leite Junior, R. P. (2013). Performance of 'Oktisu' satsuma mandarin on nine rootstocks. *Sci. Agric.* 70, 422–427. doi: 10.1590/S0103-90162013000600007

Tazima, Z. H., Neves, C. S. V. J., Yada, I. F. U., and Leite Junior, R. P. (2014). Performance of 'Oktisu' satsuma mandarin trees on different rootstocks in Northwestern Paranaì state. *Semina* 35, 2297–2308. doi: 10.5433/1679-0359.2014v35n5p2297

U.S. Department of Agriculture (1999). *Soil Taxonomy: A Basic System of Soil Classification for Making and Interpreting Soil Surveys*. Washington, DC: USDA.

Yakushiji, H., Morinaga, K., and Nonami, H. (1998). Sugar accumulation and partitioning in Satsuma mandarin tree tissues and fruit in response to drought stress. *J. Am. Soc. Hortic. Sci.* 123, 719–726. doi: 10.21273/JASHS.123.4.719

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 23 December 2021
doi: 10.3389/fpls.2021.777842

Rootstock-Mediated Genetic Variance in Cadmium Uptake by Juvenile Cacao (*Theobroma cacao* L.) Genotypes, and Its Effect on Growth and Physiology

Jessica Fernández-Paz[1,2], Andrés J. Cortés[3,4], Camila A. Hernández-Varela[1], Maria Sara Mejía-de-Tafur[2], Caren Rodriguez-Medina[1†] and Virupax C. Baligar[5†]*

[1] Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA) – C.I Palmira, Palmira, Colombia, [2] Facultad de Ciencias Agropecuarias, Universidad Nacional de Colombia Sede Palmira, Palmira, Colombia, [3] Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA) – C.I La Selva, Rionegro, Colombia, [4] Facultad de Ciencias Agrarias – Departamento de Ciencias Forestales, Universidad Nacional de Colombia Sede Medellín, Medellín, Colombia, [5] United States Department of Agriculture-Agricultural Research Service-Beltsville Agricultural Research Center, Beltsville, MD, United States

OPEN ACCESS

Edited by:
Gennaro Fazio,
Plant Genetic Resources Unit, United States Department of Agriculture-Agricultural Research Service (USDA ARS), United States

Reviewed by:
Yanhong Lou,
Shandong Agricultural University, China
Weverton Pereira Rodrigues,
Universidade Estadual da Região Tocantina do Maranhão (UEMASUL), Brazil

***Correspondence:**
Caren Rodriguez-Medina
cdrodriguez@agrosavia.co

† These authors share last authorship

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 15 September 2021
Accepted: 12 November 2021
Published: 23 December 2021

Citation:
Fernández-Paz J, Cortés AJ, Hernández-Varela CA, Mejía-de-Tafur MS, Rodriguez-Medina C and Baligar VC (2021) Rootstock-Mediated Genetic Variance in Cadmium Uptake by Juvenile Cacao (Theobroma cacao L.) Genotypes, and Its Effect on Growth and Physiology. Front. Plant Sci. 12:777842. doi: 10.3389/fpls.2021.777842

Grafting typically offers a shortcut to breed tree orchards throughout a multidimensional space of traits. Despite an overwhelming spectrum of rootstock-mediated effects on scion traits observed across several species, the exact nature and mechanisms underlying the rootstock-mediated effects on scion traits in cacao (*Theobroma cacao* L.) plants often remain overlooked. Therefore, we aimed to explicitly quantify rootstock-mediated genetic contributions in recombinant juvenile cacao plants across target traits, specifically cadmium (Cd) uptake, and its correlation with growth and physiological traits. Content of chloroplast pigments, fluorescence of chlorophyll *a*, leaf gas exchange, nutrient uptake, and plant biomass were examined across ungrafted saplings and target rootstock × scion combinations in soils with contrasting levels of Cd. This panel considered a total of 320 progenies from open-pollinated half-sib families and reciprocal full-sib progenies (derived from controlled crosses between the reference genotypes IMC67 and PA121). Both family types were used as rootstocks in grafts with two commercial clones (ICS95 and CCN51) commonly grown in Colombia. A pedigree-based best linear unbiased prediction (*A*-BLUP) mixed model was implemented to quantify rootstock-mediated narrow-sense heritability (h^2) for target traits. A Cd effect measured on rootstocks before grafting was observed in plant biomass, nutrient uptake, and content of chloroplast pigments. After grafting, damage to the Photosystem II (PSII) was also evident in some rootstock × scion combinations. Differences in the specific combining ability for Cd uptake were mostly detected in ungrafted rootstocks, or 2 months after grafting with the clonal CCN51 scion. Moderate rootstock effects ($h^2 > 0.1$) were detected before grafting for five growth traits, four nutrient uptake properties, and chlorophylls and carotenoids content ($h^2 = 0.19$, 95% CI 0.05–0.61, $r = 0.7$). Such rootstock effects faded ($h^2 < 0.1$) when rootstock genotypes were examined in soils without Cd, or 4 months after grafting. These results suggest

a pervasive genetic conflict between the rootstock and the scion genotypes, involving the triple rootstock × scion × soil interaction when it refers to Cd and nutrient uptake, early growth, and photosynthetic process in juvenile cacao plants. Overall, deepening on these findings will harness early breeding schemes of cacao rootstock genotypes compatible with commercial clonal scions and adapted to soils enriched with toxic levels of Cd.

Keywords: grafting combinations, cadmium accumulation, cadmium toxicity, rootstock mediated heritability, *Theobroma cacao*

INTRODUCTION

Grafting is an ancient technique used to propagate plants vegetatively by combining desirable agronomic traits of the rootstock with those of the scion. It has been used in several plant species targeting a wide spectrum of traits, for instance, resistance to pathogens (Boughalleb et al., 2007; Rivard and Louws, 2008; Spanò et al., 2020); tolerance to abiotic stress factors, such as water deficit, (Liu et al., 2016), heavy metals (Rouphael et al., 2008), and salinity (Yanyan et al., 2018; Suárez-Hernández et al., 2019); improved fruit quality (Davis and Perkins-Veazie, 2005); higher yields (Cardinal et al., 2007); and architectural changes in scions (Eltayb et al., 2014). However, breeding rootstocks for tree crops is slower than scion breeding for the same species. This is due to the long generation times and strong testing requirements of rootstocks, which reduce the opportunity for comprehensively testing their performance against multiple scions and environments.

Conventional propagation by rootstocks and grafting has routinely been used to expand cacao (*Theobroma cacao* L.) cultivation in tropical areas (Ríos, 1957). Seedling rootstocks resistant to *Ceratocystis* spp. and tolerant to acidic soils are typically obtained by open pollination (OP) of the IMC67, PA121, and PA46 reference genotypes in seedling orchards, and are grafted with susceptible productive clones (Palencia Calderon et al., 2007). Yet, only a few studies have evaluated the effect of the rootstock genotype on key agronomic traits such as yield and disease resistance (Yin, 2004; Ribeiro et al., 2016; Asman et al., 2021). For instance, Yin (2004) detected a significant effect of the rootstock on the vigor of cacao scions, but was unable to capture an influence on yield components such as bean weight and number of beans per pod. In line with these results, Asman et al. (2021) observed little rootstock effects on the scion's resistance to vascular streak dieback, caused by *Ceratobasidium theobromae*. On the other hand, Ribeiro et al. (2016) observed a significant influence of the rootstock × scion interaction on the scion's resistance to witches's broom disease caused by *Moniliophthora perniciosa*, which allowed the identification of an elite rootstock genotype with a positive effect on scion disease resistance trait.

Despite the long time during which grafting has been used in the vegetative propagation of *T. cacao*, the rootstock effects on the expression of key scion's agronomic traits remains poorly understood, among these, cadmium (Cd) accumulation. Content of Cd in cacao products is one of the most limiting factors for cocoa sale in international markets. Cadmium, a heavy metal that causes health problems, accumulates in the seeds of *T. cacao*, which are the raw material to produce chocolate (Barraza et al., 2017). Considering that Cd can accumulate in the human body, starting on January 1, 2019 the European Union (EU) began to control the maximum Cd concentrations allowed in chocolate and cocoa products imported to the EU (European Commission, 2014). Other countries are expected to implement similar regulations to Cd concentration in cocoa products (Vanderschueren et al., 2021), which has generated concern in countries where levels of bioavailable Cd for the plant have been detected in soil. Particularly, high Cd concentrations in cacao beans have been reported in plantations from South America (Chavez et al., 2015; Arévalo-Gardini et al., 2017; Vanderschueren et al., 2019), which has been correlated with the naturally high Cd content in the young soils of this region (Argüello et al., 2019). The Cd concentration in cacao bean is related to soil properties such as total soil Cd, pH, percentage of organic carbon, and oxalate extractable manganese (Argüello et al., 2019). Cd adversely affects growth, photosynthetic process, nutrient uptake, content of chloroplast pigments, cell structure, antioxidative metabolism, and gene expression (Dias et al., 2012; Jiao et al., 2012; Borek et al., 2013; Saidi et al., 2013; Castro et al., 2015; Farooq et al., 2016; Pereira de Araújo et al., 2017).

Cadmium enters root cells through ion channels for Zn, Fe, Mn, and Ca (Song et al., 2017). Transporter gene families like zinc-iron permease (ZIP), natural resistance-associated macrophage proteins (NRAMPs), and heavy metal transporting ATPases (HMAs) have been associated with uptake and translocation of Cd in plants (Vanderschueren et al., 2021). According to some studies, TcNRAMP5 may play a role in the regulation of Cd uptake in cacao plants (Ullah et al., 2018; Moore et al., 2020), whereas HMA-family proteins may contribute to Cd sequestration (Moore et al., 2020). However, the information regarding the specific role of transporter genes in cacao is still limited, as well as the regulatory mechanisms of Cd translocation to the shoot. Within the roots, Cd can be transported in plants through a symplasmic pathway. Cd is later loaded from the symplasm into the xylem (Lux et al., 2011). Movement of Cd from the roots to above-ground tissues depends on the plant mechanisms for vacuolar sequestration, xylem loading, and xylem to phloem transfer (Vanderschueren et al., 2021). In soybean and eggplant, the effect of the root system on the accumulation of Cd in the aerial part of the plant has been demonstrated by grafting cultivars on genotypes that differ in their accumulation of Cd (Sugiyama et al., 2007; Arao et al., 2008). The feasibility of mitigating Cd uptake and accumulation through grafting of highly productive scions on top of rootstocks

with low Cd uptake capability has been suggested for *T. cacao* (Lewis et al., 2018; Engbersen et al., 2019). However, as far as we are aware, such effect has not yet been experimentally demonstrated. Further investigation is needed to elucidate the effect of the rootstock in Cd accumulation in cacao. Rootstock genotype may produce unpredicted responses in grafted plants exposed to stressful Cd conditions compared with self-rooted plants (Savvas et al., 2010).

Assessing the effect of rootstock genotypes or specific rootstock × scion combinations on the accumulation of Cd in plant tissues, and the overall plant tolerance to heavy elements, is therefore key for the establishment of new cacao plantations in regions with high levels of available Cd in soil. Therefore, the aim of this study was to quantify the inheritance of the rootstock effects on the scion's Cd accumulation, as well as associated growth and physiological traits of juvenile cacao plants. Both open-pollinated (OP) and reciprocal full-sib progenies (derived from controlled crosses between the reference genotypes IMC67 and PA121) were used as rootstocks in grafts with two commercial and widely adapted clones (ICS95 and CCN51) in Colombia. Content of chloroplast pigments, fluorescence of chlorophyll *a*, leaf gas exchange, ion leakage, protein content, nutrient uptake, and plant biomass were also examined across rootstock × scion combinations in soils with contrasting levels of Cd. We hypothesize that due to the complex acquisition and transportation of Cd from the root system throughout the plant, there is a potential to harness rootstock-driven genetic variance for Cd uptake as part of early selection schemes at seed orchards, nurseries, and ungrafted saplings.

MATERIALS AND METHODS

Location

The experiment was established under greenhouse conditions at the Palmira Research Station of AGROSAVIA, located in Palmira (3°31′12″N, 76°19′50″W), province of Valle del Cauca, Colombia, at an altitude of 1,001 masl. The climatic conditions during the experiment were recorded using a weather station WatchDog 1000 series Micro Station. Average annual temperature was 26°C, and average relative humidity was 65%.

Soil Substrate

Soil collected in a cacao growing region of southwest Colombia (pH 4.5) had a natural total Cd content of 0.43 mg kg^{-1}. The collected soil was air-dried and passed through a 2-mm mesh sieve. Cd was added to half of the sieved soil using an aqueous solution of $Cd(NO_3)_2$. Cd-spiked soil was incubated at field capacity for 1 month in a greenhouse. During the incubation time, the soil was constantly mixed and maintained at field capacity. The other half of the sieved soil was kept untouched as control treatment.

Greenhouse substrate was prepared by mixing Cd-spiked soil with rice husk and sand in a ratio of 3:1:1 to reach a final content of 7.49 mg Cd kg^{-1}, as determined by inductively coupled plasma optical emission spectrometry (Thermo Scientific ICAP 6500). Greenhouse substrate was also prepared using soil without the addition of Cd. According to soil analysis, carried out before the establishment of the experiment, the total nitrogen content in the substrate was 0.4 and 0.5% for soil without addition of Cd and soil enriched with Cd, respectively, which shows that there was no advantage in the concentration of nitrogen in the treatment with addition of Cd.

Plant Material

Plant material was obtained from the Colombian Cacao Germplasm Bank. Full-sib progenies obtained from the crossing between IMC67 × PA121, and its reciprocal cross, were established in Cd-spiked soil, as well as in soil without addition of Cd. Progenies were subsequently evaluated for plant growth, and physiological and nutritional parameters. Meanwhile, a total of two OP half-sib seedling families of IMC67 and PA121 were also considered. These accessions, PA121 and IMC67, corresponded to genotypes recommended as rootstocks in Colombia due to their resistance to pathogens and adaptation to soils with acidic pH (Palencia Calderon et al., 2007). The mucilage of the seed was removed by gently rubbing with sawdust. Seeds were sown in plastic conical tubes containing sand and grown under these conditions for 2 months. After this period, well-developed seedlings were transplanted into black polyethylene bags of 45 cm high and 29 cm wide, containing 10 kg greenhouse substrate with and without the addition of Cd. A commercial fertilizer containing N, P, K, and Mg was applied.

The experiment was established in a randomized complete block design with four replicates arranged in a 4 × 2 factorial design and 10 plants per experimental unit. The treatments corresponded to OP families IMC67 and PA121, as well as IMC67 × PA121 and PA121 × IMC67 progenies from controlled crosses established in soil with and without the addition of Cd. Seedlings were grown on the Cd treatments for 5 months and then evaluated for plant growth, physiological, and nutritional parameters at the end of this period.

Five months after Cd treatment, budwoods of the commercial cultivars ICS95 and CCN51 were grafted onto the seedling rootstocks using the top grafting technique as described by Isele et al. (2020). Taking into consideration that each experimental unit had 10 plants, two of each were evaluated without grafting, four of the remaining plants were grafted with ICS95, and the other four with CCN51. Two and 4 months after grafting, rootstock × scion combinations were evaluated for plant growth, physiological, and nutritional parameters.

Chlorophyll Fluorescence

Chlorophyll fluorescence was determined by means of a portable optical pulse fluorometer (Opti-Sciences OS30P+). The measurements were made between 7:00 and 11:00 on young, fully expanded, healthy, and photosynthetically active leaves of two plants per experimental unit, usually the third or fourth leaf from the apex of the plants. The leaf was adapted to darkness, using suitable clips, for a period of 30 min and subsequently illuminated with a saturating actinic light pulse of 3,500 $\mu mol\ m^{-2}\ s^{-1}$ for 1 s. The following parameters were evaluated: initial fluorescence (F_0), maximum fluorescence (F_m), and the maximum quantum yield of PSII (F_v/F_m), obtaining two data per plant.

Leaf Gas Exchange

Net photosynthetic rate (P_N), stomatal conductance to water vapor (g_s), leaf transpiration (E), internal carbon dioxide concentration (Ci), and instantaneous water use efficiency (WUE_{INST}) were measured on the same leaf used to determined chlorophyll fluorescence. Gas exchange measurements were performed between 7:00 and 11:00 using an open system portable gas analyzer (ADC model LCpro+), natural light intensity between 250 and 450 μmol m^{-2} s^{-1} and atmospheric CO_2 of 390 μmol (CO_2) mol^{-1}. The leaf area sampled was 6.25 cm^2. Two data points per plant were obtained.

Chloroplast Pigments

Chlorophyll *a*, chlorophyll *b*, their ratio, total chlorophyll, and carotenoids were quantified in the same leaves used to measure gas exchange and fluorescence emission. Pigments were extracted from three disk-shaped leaf segments per plant using cold 80% ammoniacal acetone (4°C), following the protocol described by Melgarejo et al. (2010) and calculated according to Lichtenthaler (1987). The area of each disk was 0.78 cm^2. The absorbance of the extracts was determined with a spectrophotometer (PerkinElmer Lambda 25) using the following wavelengths: 663, 647, and 470 nm to calculate the concentrations of chlorophyll *a*, chlorophyll *b*, and total carotenoids, respectively (Lichtenthaler, 1987).

Pigment concentration was calculated from the following equations:

$$\mathrm{Ca} = 12.25\ \mathrm{A}_{663-2.79}\ \mathrm{A}_{647}$$

$$\mathrm{Cb} = 21.5\ \mathrm{A}_{647-5.1}\ \mathrm{A}_{663}$$

$$\mathrm{C}\left(\mathrm{a}+\mathrm{b}\right) = 7,15\ \mathrm{A}_{663+18.7}\ \mathrm{A}_{647}$$

$$\mathrm{Cx}+\mathrm{c} = 1000\ \mathrm{A}_{470-1.82}\,\mathrm{Ca} - 85,02\,\mathrm{Cb}/198$$

where Ca stands for chlorophyll *a*, Cb stands for chlorophyll *b*, C (a + b) stands for total chlorophyll, and Cx + c stands for carotenoids.

Electrolyte Leakage

To determine electrolyte leakage (EL), 0.78 cm^2 leaf disks were taken from the same leaf used to determine the content of chloroplast pigments. Three disks were obtained from each leaf sample, washed with distilled water, and then arranged in 15 mL conical Falcon tubes containing 3 mL of deionized water. The samples were incubated at room temperature (22°C) on a shaker for a period of 5 h and the electrical conductivity was recorded at the end of this period (EC1). The Falcon tubes were then taken to a water bath at 80°C for 10 min and electrical conductivity (EC2) recorded again after cooling the bathing solution to 22°C. The loss of electrolytes (EL) was calculated using the following formula:

$$EL = \frac{EC1}{EC2} \times 100$$

EC1 corresponds to the electrical conductivity measured after 5 h in deionized water. EC2 corresponds to the electrical conductivity measured after incubation at 80°C.

Plant Growth and Biomass

Once the physiological parameters were evaluated during morning hours, seedling rootstocks, and also grafting combinations, were harvested for shoot and root lengths measures. Plants were then divided into roots, stem, and leaves. Plant tissue was washed with distilled water and gently dried with absorbent towels. Fresh weight of each plant portion was then determined. To wash the excess of Cd adhered to the roots, these were immersed for 3 min in a solution containing 5 mM EDTA and 20 mM TRIS (pH 8.0) with constant agitation followed by three washes with distilled water for 3 min (Nguyen et al., 2016). The reason why roots were washed was to prevent externally adhered Cd from being quantified as if it were inside the root system. Samples were placed in paper bags and oven-dried at 60°C for 72 h, and after this period dry weight was determined.

Leaf Area

Leaf area was measured using an electronic area meter (LICOR-3000) immediately after harvesting leaf tissue.

Protein Content

Approximately 100 mg of leaf tissue were macerated in liquid nitrogen. Phosphate buffer (0.05 M) was then added, and the samples centrifuged for 30 min at 12,000 × *g* and 4°C. Total soluble protein content was measured by the Coomassie blue method according to Bradford (1976) using BSA as standard in a microplate spectrophotometer (BioTek EPOCH) at 595 nm.

Cadmium and Mineral Nutrients Content

Dried plant material was ground in a mill (Thomas Wiley) and submitted to nitric–perchloric digestion assisted by microwave (Milestone UltraWave). Mineral elements such as Cd, Ca, Mg, Fe, Zn, Cu, and Mn were determined by atomic absorption spectrophotometry, P by colorimetry, and K by flame emission photometry (Isaac and Kerber, 1971). Nitrogen content was determined by the Kjeldahl method (Bradstreet, 1954).

Data Analyses and Rootstock-Mediated Heritability Scores

Trait variation across families was explicitly compared using a mixed linear model in which family identity and Cd treatment were indexed as fixed effect and repetition as random effect. Second, rootstock-mediated additive genetic variance (i.e., narrow sense hereditability) was obtained for each trait using a best linear unbiased prediction (BLUP) mixed model that relied on the pedigree information (i.e., a pedigree relationship matrix for an *A*-BLUP model) from the rootstocks (half-sib families for OP, and full-sib families for controlled crossing schemes, allowing for a 5% of maternal effects). The corresponding linear mixed model, following Arenas et al. (2021), was fitted as in:

$$y = Xb + Za + e$$

where y is the phenotypic trait vector, a is a vector of individual random additive genetic effects with a normal distribution so that $a \sim N(0, A\sigma_a^2)$ with A being the pedigree relationship matrix among rootstocks and σ_a^2 the additive genetic variance (Müller et al., 2017), b is a vector of fixed effects (i.e., intercept or general mean, and experimental site effects), e is the vector of residual error effect, and X and Z are the corresponding incidence matrices for fixed effects and additive genetic effects (Chen et al., 2018; Gutierrez et al., 2018), respectively.

To aid interpretability while accounting for clonal scion differences and *in situ* environmental (soil) variation (of major importance in Cd fixation), genetic parameters for all phenotypic traits were individually estimated for each environment and scion, rather than including the latter as explicit fixed effects within the model. All models implemented the reproducing kernel Hilbert space (RKHS) method throughout the BGLR (Bayesian Generalized Linear Regression) package (Pérez-Rodríguez and de los Campos, 2014) in R v.3.4.4 (R Core Team). RKHS is a semiparametric approach to infer a given function without making a strong *a priori* assumption about the distribution of effects (Cuevas et al., 2016). RKHS was executed using a Gibbs sampling with 10,000 iterations after discarding the 5,000 initial steps as burn-in. A thinning interval of 10 was implemented for data recording. Convergence of posterior distributions was verified using trace plots, whereas rootstock-mediated specific combining abilities, original trait correlations (**Supplementary Figure 1**), and A input pedigree relationship matrices (**Supplementary Figure 2**) were respectively checked using the R (R Core Team) functions *lme* (from the *nlme* package, treating families as fixed effects and repetitions as random effects), *cor.test*, and *heatmap*.

Narrow sense rootstock-mediated heritability (h^2) was then computed using the additive (σ_a^2) and residual (σ_e^2) variances, following de los Campos et al. (2015), as in:

$$h^2 = \sigma_a^2 / \left(\sigma_a^2 + \sigma_e^2\right)$$

Variance within experimental units was captured by the residual variance term because it did not correspond to an additive variance component. Narrow sense rootstock-mediated heritability (h^2) estimates were summarized using the median and the 95% confidence interval from the BGLR's posterior distribution. Overall models' fits were examined by computing the prediction ability (r_y) estimated for each trait as the Pearson's correlation between the observed and the predicted (i.e., breeding value deviation from the overall mean) trait values (Müller et al., 2017; Zhang et al., 2019).

RESULTS

Cadmium Accumulation

Significant differences in the accumulation of Cd in roots before grafting were observed between the seedling rootstocks established in Cd-spiked soil. Accumulation of Cd in the roots of PA121 × IMC67 full-sib progenies was significantly higher ($p < 0.05$) than in IMC67 OP half-sib and IMC67 × PA121 full-sib families (**Figure 1A**). On the other hand, no significant differences were observed in the accumulation of Cd in leaves between the family rootstocks subjected to stress by Cd (**Figure 1B**).

Cadmium accumulation in both rootstocks and scions was quantified 2 and 4 months after grafting the ICS95 and CCN51 clonal scions on the seedling rootstock families (**Figure 2**). At 2 months after grafting, lower accumulation of Cd in leaf tissue was observed when the ICS95 clone was grafted on the IMC67 OP half-sib progenies (**Figure 2A**). However, no significant differences in Cd accumulation were observed between any of the rootstock families on which ICS95 was grafted (**Figure 2C**). Four months after grafting, a greater accumulation of Cd in leaf tissue of the ICS95 clonal scion grafted on PA121 × IMC67 full-sib rootstocks was observed (**Figure 2A**). For this case, lower accumulation of Cd in roots of the IMC67 OP half-sib and IMC67 × PA121 full-sub families was observed when grafted with the ICS95 clonal scion (**Figure 2C**).

On the other hand, no significant differences were observed in Cd accumulation of the CCN51 clonal scion when grafted on any of the evaluated rootstocks 2 months after grafting (**Figure 2B**). However, a greater accumulation of Cd was observed in PA121 × IMC67 full-sib progenies when they were grafted with the CCN51 scion (**Figure 2D**). Four months after grafting, a greater accumulation of Cd was observed in CCN51 clonal scion grafted on IMC67 × PA121 full-sib rootstocks, and its reciprocal progenies (**Figure 2B**). For this case, no significant differences in Cd accumulation were observed between rootstock families having CCN51 as scion (**Figure 2D**).

Cadmium accumulation in the leaf tissue of the CCN51 scion grafted on IMC67 OP half-sib rootstock progenies, 2 and 4 months after grafting, was significantly higher than for the ICS95 clonal scion (**Figure 3A**). No significant differences in Cd accumulation were observed between ICS95 and CCN51 when grafted onto any of the other rootstock families at 2 or 4 months after grafting (**Figure 3A**). Significant differences in Cd accumulation in the roots were observed only for the PA121 × IMC67 full-sib rootstock family. A higher accumulation of Cd was observed when this rootstock family was grafted with the CCN51 scion 2 months after grafting. However, 4 months after grafting a higher accumulation of Cd in the roots was observed when using ICS95 as scion (**Figure 3B**).

Plant Growth

A significant decrease in plant biomass, shoot length, and leaf area was observed 5 months after establishment of the ungrafted seedlings in Cd-spiked soil (**Figures 4A–F**). Fresh weight of the stem decreased by 13.7, 37.6, 8.3, and 13.2% for the OP half-sib families IMC67 and PA121, and for the full-sib IMC67 × PA121 and PA121 × IMC67 progenies, respectively, as compared with the control (without Cd addition) (**Figure 4A**). A greater decrease in stems' (37.4%) and roots' (19.5%) dry weight was also observed for PA121 OP half-sib rootstocks subjected to stress by Cd addition in the soil (**Figures 4B,D**). A similar response was observed for shoot length, for which a decrease of 15.8, 32, 13.6, and 2.8% was observed respectively, for OP half-sib

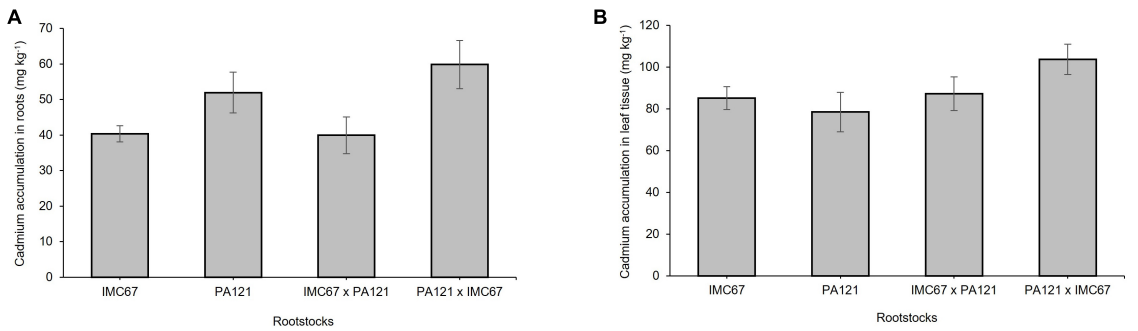

FIGURE 1 | Cadmium accumulation across four ungrafted seedling rootstocks grown in soil enriched with Cd (7.49 mg Cd kg^{-1} soil) 5 months after treatment. **(A)** Cadmium accumulation in roots. **(B)** Cadmium accumulation in leaves. Data are mean values ± SE (n = 8).

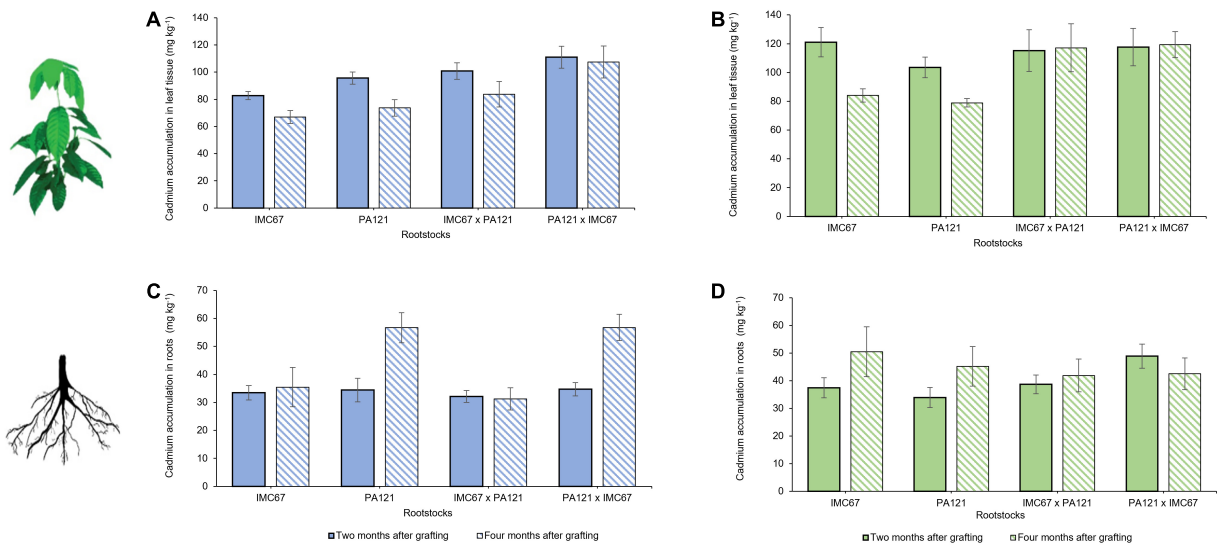

FIGURE 2 | Cadmium accumulation in rootstock × scion combinations 2 and 4 months after grafting. Seedling rootstocks families were established in soil enriched with Cd (7.49 mg Cd kg^{-1} soil). Panels **(A,C)** depict cadmium accumulation in grafting combinations having ICS95 as scion. Panels **(B,D)** show Cd accumulation in grafting combinations having CCN51 as scion. Data are mean values ± SE (n = 8).

progenies IMC67 and PA121, and full-sib IMC67 × PA121 and PA121 × IMC67 families (**Figure 4E**).

Two months after grafting, significant changes in root biomass were observed for grafting combinations having both ICS95 and CCN51 as scion (**Figures 5A–D**). Combinations of ICS95 as scion and PA121 half-sib progenies as rootstock showed a significant decrease in root biomass. Specifically fresh and dry root weight fell in 25.5 and 24.6%, respectively, compared with the control (**Figures 5A,C**). Likewise, 2 months after grafting, PA121 half-sib rootstock families showed a significant decrease in root biomass in combination with the CCN51 clonal scion. For this case, fresh and dry root weight respectively, decreased by 35.13 and 36.15% compared with the control (without Cd addition) (**Figures 5B,D**).

Four months after grafting, a significant decrease in root biomass was also observed in all rootstock families grafted with the ICS95 scion (**Figures 6A,C**). On the other hand, 4 months after grafting, seedling rootstocks grafted with the CCN51 clonal scion showed significant changes not only in root growth parameters but also in the scion growth. A significant decrease in leaf area and in fresh and dry weight of leaf tissue was observed in the CCN51 scion grafted on all the rootstock families, except when it was grafted on PA121 × IMC67 full-sib rootstocks (**Figures 6B,D**). A decrease in dry root weight of 23.5 and 37.2% was respectively, observed in the OP half-sib rootstock families IMC67 and PA121, both grafted with the CCN51 clonal scion (to be presented in section Rootstock-Mediated Specific Combining Abilities and Heritability Scores in Full-Sib Families).

Chlorophyll Fluorescence and Leaf Gas Exchange

A decrease in the net photosynthetic rate (P_N) and in instantaneous water use efficiency (WUE_{INST}) was observed in response to Cd toxicity before grafting (**Figure 7**). A significant decrease ($p < 0.05$) in the carbon assimilation rate equivalent

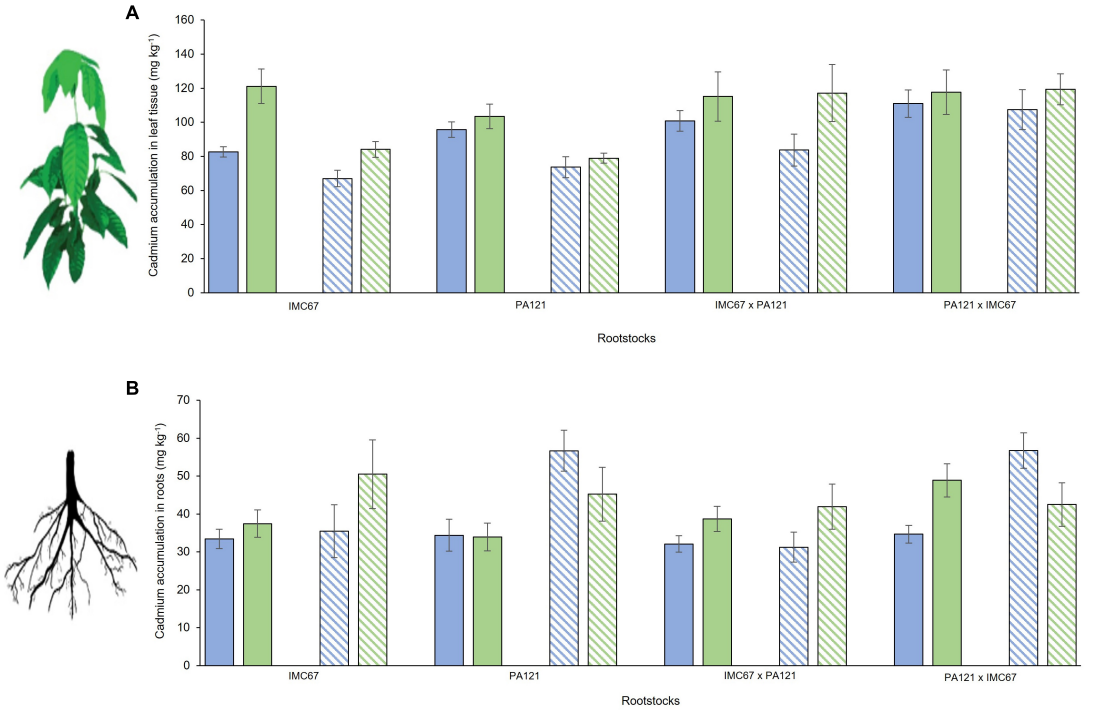

FIGURE 3 | Cadmium accumulation of ICS95 and CCN51 clonal scions grafted on four seedling rootstock families 2 and 4 months after grafting. Seedling rootstocks (*x*-axis) were grown in soil enriched with Cd (7.49 mg Cd kg^{-1} soil). **(A)** Cadmium accumulation in leaf tissue of the ICS95 and CCN51 clonal scions. **(B)** Cadmium accumulation in the roots of the four OP rootstock families when grafted with the ICS95 and CCN51 clones. Blue bars represent grafting combinations with ICS95 as scion 2 months after grafting. Green bars represent grafting combinations with CCN51 as scion 2 months after grafting. Data are mean values ± SE (n = 8). Dashed bars show Cd accumulation 4 months after grafting.

to 40.09%, compared with the control, was observed when using PA121 OP half-sib seedling rootstocks (**Figure 7A**). Water use efficiency respectively, decreased by 30.46 and 40.6% when using IMC67 and PA121 OP half-sib seedling rootstock families, compared with control plants (**Figure 7B**). There was no effect of Cd on leaf gas exchange variables when using ICS95 and CCN51 as clonal scions, neither at 2 nor at 4 months after grafting.

Variables of chlorophyll *a* fluorescence emission did not show significant differences ($p < 0.05$) between seedling rootstock families established in soil with and without the addition of Cd before grafting (to be presented in section Rootstock-Mediated Specific Combining Abilities and Heritability Scores in Full-Sib Families). However, 2 months after grafting, a significant decrease in F_m was observed in ICS95 clonal scion grafted on all the rootstock families. A greater decrease of 19.4% in F_m was observed in this clonal scion grafted on IMC67 × PA121 full-sib rootstock progenies (**Figure 8A**). An effect of Cd on F_m resulted in a change in F_v/F_m ratio scores. A significant decrease in F_v/F_m was observed in the ICS95 clonal scion grafted on PA121 OP half-sib and IMC67 × PA121 full-sib rootstock families 2 months after grafting (**Figure 8B**). On the other hand, an increase in F_v/F_m ratio was observed in the same clone when grafted on the reciprocal PA121 × IMC67 full-sib progenies 2 and 4 months after grafting (**Figures 8B,C**), which suggests for a maternal effect. There was no significant difference when the clonal scion CCN51 was grafted with the different seedling rootstock families for the variables of chlorophyll fluorescence at 2 or at 4 months after grafting (to be presented in section Rootstock-Mediated Specific Combining Abilities and Heritability Scores in Full-Sib Families).

Chloroplast Pigments

Significant changes in the content of chloroplast pigments were observed in the ungrafted seedlings 5 months after establishment in Cd-spiked soil (**Figure 9**). A different effect of Cd was observed depending on the rootstock. A significant increase in chlorophyll *a* equivalent to 41.6 and 21.8% was respectively, observed in IMC67 OP half-sib and PA121 × IMC67 full-sib progenies, compared with the control (**Figure 9A**). IMC67 OP half-sib and PA121 × IMC67 full-sib families also showed a significant increase in chlorophyll *b* content respectively, equivalent to 32 and 29% (**Figure 9B**). Changes in chlorophyll *a* and *b* resulted in changes in total chlorophyll, accordingly. A significant increase of total chlorophyll was observed in IMC67 OP half-sib and PA121 × IMC67 full-sib families (**Figure 9C**). The carotenoid content also increased by 17% in IMC67 OP half-sib progenies 5 months after Cd addition. The content of carotenoids in the remaining families decreased (**Figure 9D**).

Two months after grafting, a significant decrease in all chloroplast pigments was observed across seedling rootstock families grafted with both ICS95 and CCN51 clonal scions (**Figure 10**).

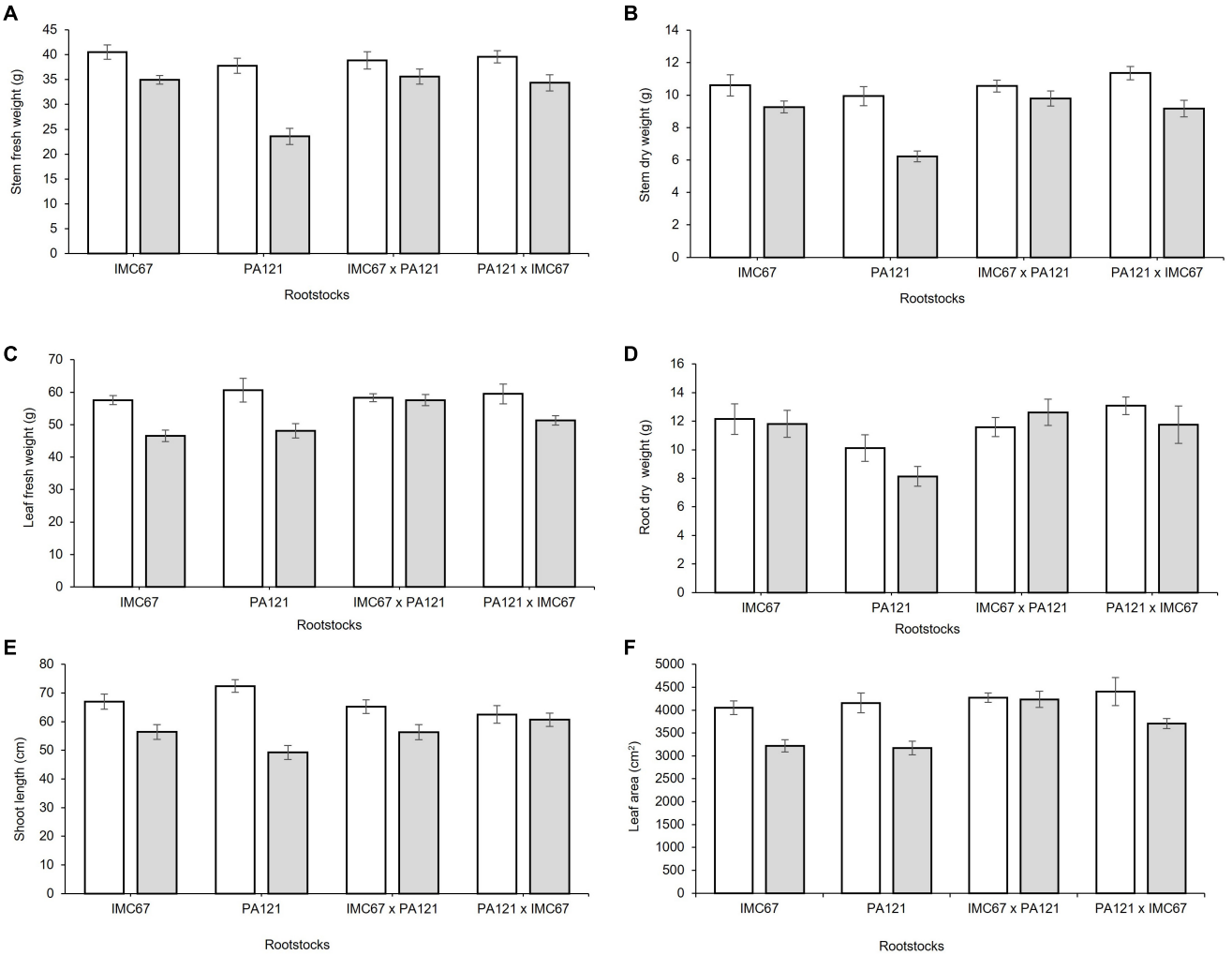

FIGURE 4 | Plant growth of four families of ungrafted seedlings under greenhouse conditions at two different concentrations of Cd in soil, 5 months after cadmium treatment. **(A)** Stem fresh weight, **(B)** stem dry weight, **(C)** leaf fresh weight, **(D)** root dry weight, **(E)** shoot length, and **(F)** leaf area. White bars represent rootstocks grown in soil without addition of Cd (0.43 mg kg^{-1}). Gray bars represent rootstocks grown in Cd spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

Electrolyte Leakage

A significant increase of 24.7 and 30.6% in electrolyte leakage was observed in ungrafted IMC67 and PA121 OP half-sib families established in Cd-spiked soil, suggesting cell membrane damage in these rootstocks (**Figure 11**). No significant difference was observed in electrolyte leakage after grafting with the ICS95 and CCN51 clonal scions in Cd-spiked soil (**Supplementary Table 5**).

Protein Content

A decrease of 22.2% in total soluble protein content was observed in PA121 OP half-sib seedlings before grafting (**Figure 12A**). Two months after grafting, a significant decrease in total soluble protein content of 39.5 and 38.5% was observed respectively in the ICS95 clonal scion grafted on the IMC67 OP half-sib, and IMC67 × PA121 full-sib families, compared to the control plants without Cd addition (**Figure 12B**). A significant decrease in protein content in leaf tissue of the CCN51 clonal scion grafted on IMC67 OP half-sib progenies, equivalent to 31% compared with the control, was also observed 2 months after grafting (**Figure 12C**).

Mineral Nutrients Content in Leaf Tissue

Cadmium absorption resulted in changes in the accumulation of mineral elements in leaf tissue of ungrafted seedling (**Supplementary Table 1**). Significant increases of 14 and 17.3% in K content were observed in PA121 OP half-sib and PA121 × IMC67 full-sib families compared with the control. N content decreased in all seedling families, while Mg content increased. However, these changes were significant only in IMC67 × PA121 full-sib progenies, compared with the control (without Cd addition). A significant increase in P content in leaf tissue was observed only in IMC67 × PA121 full-sib family established in Cd-spiked soil. In relation to micronutrients, changes in Fe content in leaf tissue were significant only in

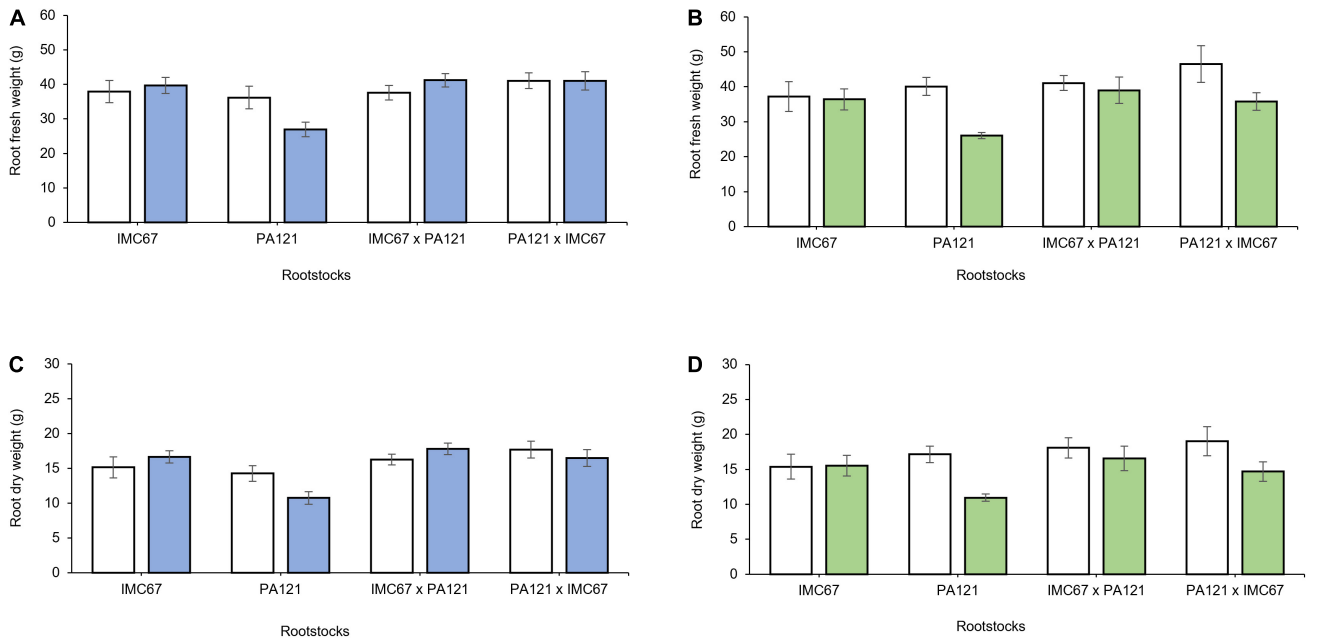

FIGURE 5 | Root biomass of rootstock × scion combinations 2 months after grafting seedling rootstocks subjected to two different concentrations of Cd in soil under greenhouse conditions. Panel **(A,B)** depict fresh root weight of rootstock families having ICS95 and CCN51 as scions. Panel **(C,D)** show dry root weight of OP and full-sib rootstock progenies having ICS95 and CCN51 as scion. White bars represent rootstocks grown in soil without addition of Cd (0.43 mg kg^{-1}). Blue bars represent rootstocks grown in Cd spiked soil (7.49 mg kg^{-1}) grafted with the ICS95 clonal scion. Green bars represent rootstocks grown in Cd spiked soil (7.49 mg kg^{-1}) grafted with the CCN51 clonal scion. Data are mean values ± SE (n = 8).

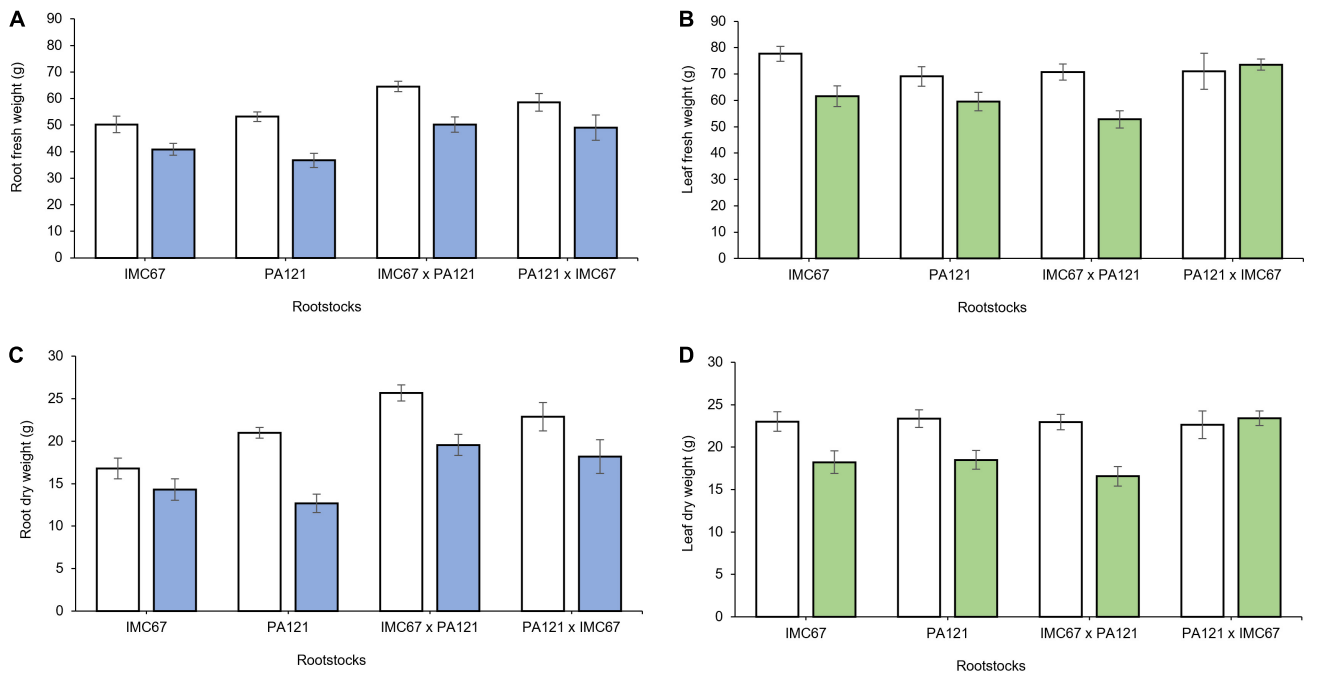

FIGURE 6 | Plant growth of rootstock × scion combinations 4 months after grafting at two different concentrations of Cd in soil under greenhouse conditions. Panels **(A,C)** show fresh and dry root weight of seedling rootstocks grafted with the ICS95 clonal scion. Panels **(B,D)** depict fresh and dry leaf weight of the CCN51 clonal scion across rootstock families. White bars represent rootstocks grown in soil without addition of Cd (0.43 mg kg^{-1}). Blue bars represent rootstock families grafted with the ICS95 clonal scion established in Cd spiked soil (7.49 mg kg^{-1}). Green bars represent rootstock families grafted with the CCN51 clonal scion established in Cd spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

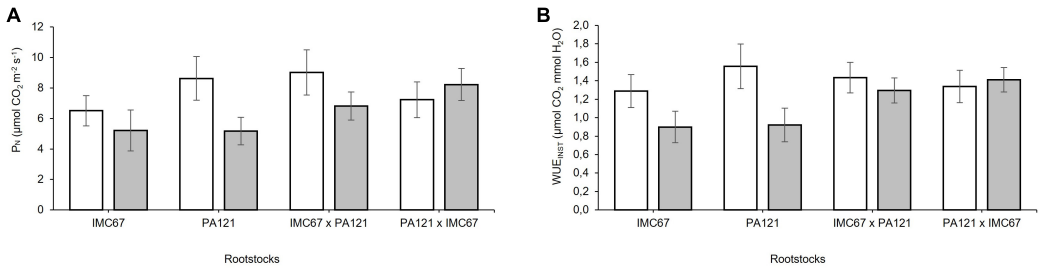

FIGURE 7 | Leaf gas exchange in four seedling rootstock families subjected to two different concentrations of Cd in soil under greenhouse conditions 5 months after cadmium treatment. **(A)** Photosynthetic rate (P_N), and **(B)** instantaneous water use efficiency (WUE_{INST}). White bars represent rootstock families grown in soil without addition of Cd (0.43 mg kg^{-1}). Gray bars represent rootstock families grown in Cd spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

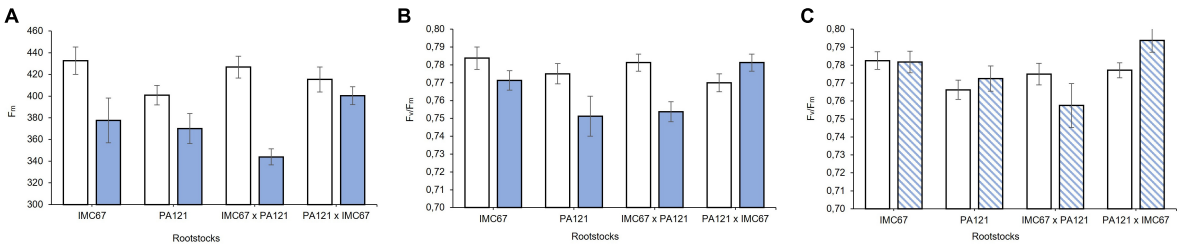

FIGURE 8 | Chlorophyll fluorescence in the ICS95 clonal scion when grafted with 4 different rootstock families at two different concentrations of Cd in soil under greenhouse conditions. **(A)** F_m 2 months after grafting. **(B)** F_v/F_m 2 months after grafting. **(C)** F_v/F_m 4 months after grafting. White bars represent rootstock families grown in soil without addition of Cd (0.43 mg kg^{-1}). Blue bars represent rootstock families grown in Cd spiked soil (7.49 mg kg^{-1}). Dashed bars show Cd accumulation 4 months after grafting. Data are mean values ± SE (n = 8).

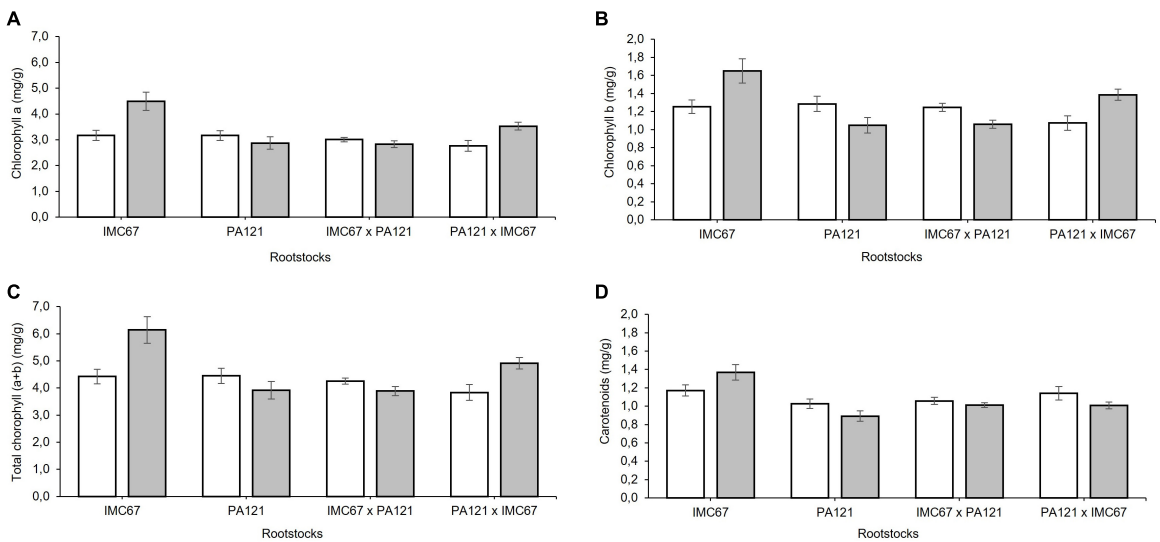

FIGURE 9 | Content of chloroplast pigments of ungrafted seedling families subjected to two different concentrations of Cd in soil under greenhouse conditions 5 months after cadmium addition. **(A)** Content of chlorophyll *a*. **(B)** Content of Chlorophyll *b*. **(C)** Content of total Chlorophyll. **(D)** Content of carotenoids. White bars represent seedling families grown in soil without addition of Cd (0.43 mg kg^{-1}). Gray bars represent seedling progenies grown in Cd spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

IMC67 OP half-sib progenies, for which there was a reduction of 16.3% in relation to the control. Zn and Mn content decreased in all seedling families subjected to stress by Cd. A significantly higher decrease in the Zn content of 25.8% was observed in PA121 OP half-sib progenies, compared with the control. Mn content respectively decreased by 10.9, 24.4, 11.5, and 21.5% for PA121 and IMC67 OP half-sib, and PA121 × IMC67 and IMC67 × PA121 full-sib families, in relation to the control.

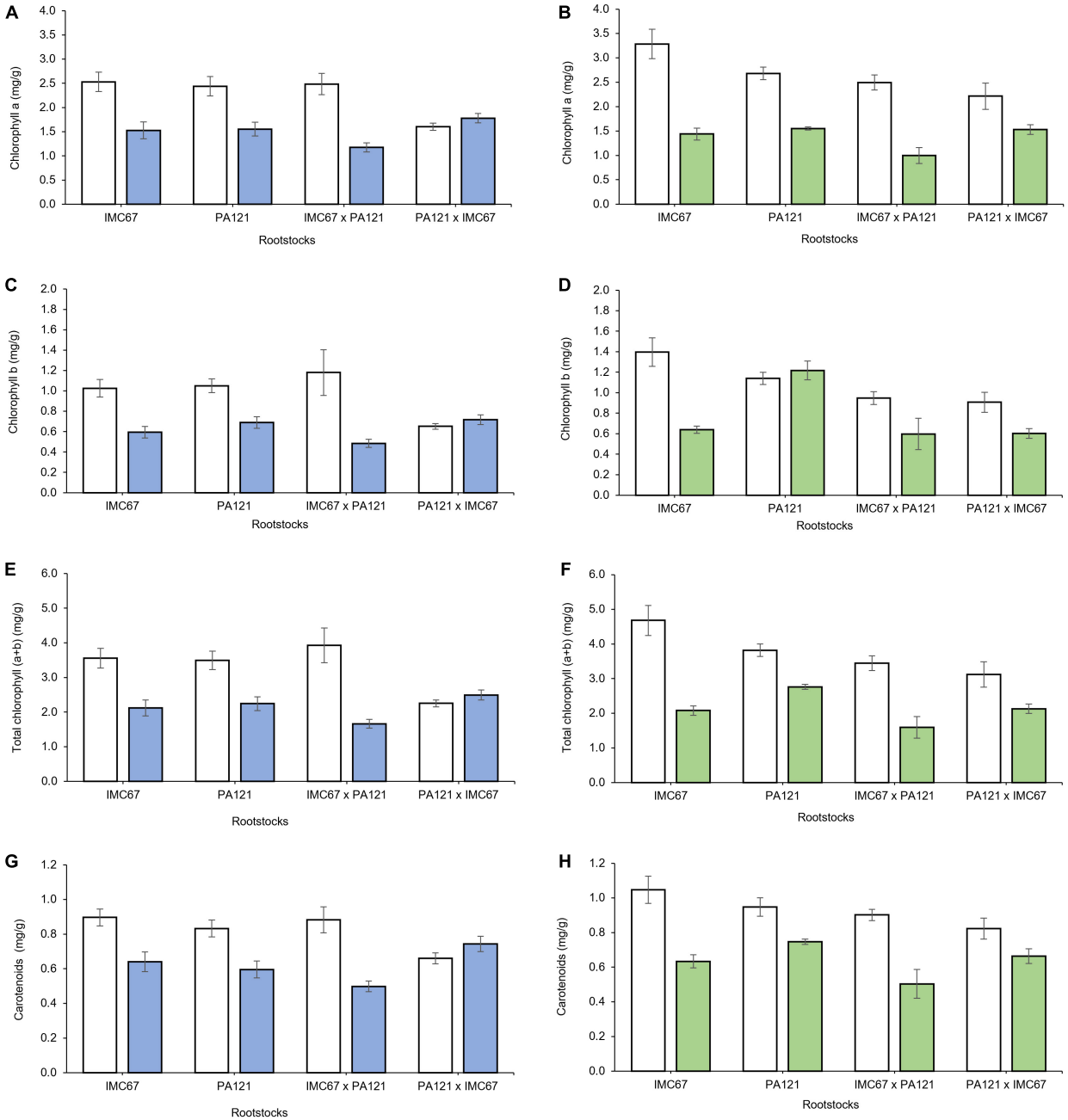

FIGURE 10 | Content of chloroplast pigments in ICS95 and CCN51 clonal scions 2 months after grafting on four different seedling rootstock families subjected to two different concentrations of Cd in soil under greenhouse conditions. Panels **(A,B)** show content of Chlorophyll a of ICS95 and CCN51 clonal scions. Panels **(C,D)** depict content of Chlorophyll b of ICS95 and CCN51 clonal scions. Panels **(E,F)** exhibit content of total Chlorophyll of ICS95 and CCN51. Panels **(G,H)** show content of carotenoids of ICS95 and CCN51 clonal scions. White bars represent rootstocks × scion combinations grown in soil without addition of cadmium (0.43 mg kg^{-1}). Blue bars show seedling rootstock families grafted with the ICS95 clonal scion grown in Cd spiked soil (7.49 mg kg^{-1}). Green bars depict seedling rootstock families grafted with the CCN51 clonal scion grown in Cd spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

Mineral elements concentration in leaf tissue of clonal scions across seedling rootstock families is shown in **Supplementary Table 2**. Two months after grafting, significant changes were observed in the concentrations of macro- and microelements in both CCN51 and ICS95.

Rootstock-Mediated Specific Combining Abilities and Heritability Scores in Full-Sib Families

In ungrafted full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, specific

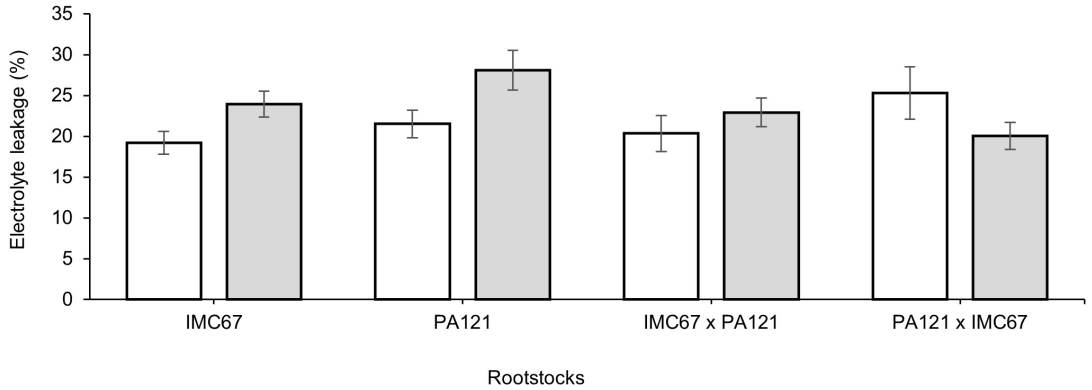

FIGURE 11 | Electrolyte leakage in four ungrafted seedling families subjected to two different concentrations of Cd in soil under greenhouse conditions 5 months after Cd addition. White bars represent ungrafted seedling families grown in soil without the addition of Cd (0.43 mg kg^{-1}). Gray bars represent ungrafted seedling families grown in Cd-spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

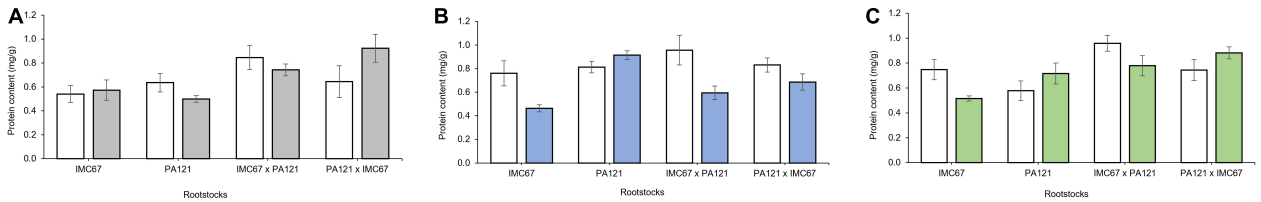

FIGURE 12 | Protein content in leaf tissue of rootstocks and grafting combinations subjected to two different concentrations of Cd in soil under greenhouse conditions. **(A)** Protein content in leaf tissue of ungrafted seedling families 5 months after Cd addition. **(B)** Protein content in leaf tissue of the ICS95 clonal scion 2 months after grafting with four different rootstock families. **(C)** Protein content in leaf tissue of the CCN51 clonal scion 2 months after grafting with four different rootstock families. White bars represent families grown in soil without addition of Cd (0.43 mg kg^{-1}). Gray bars represent ungrafted seedling families grown in Cd-spiked soil (7.49 mg kg^{-1}). Blue bars represent seedling rootstock families grafted with the ICS95 clonal scions in Cd-spiked soil (7.49 mg kg^{-1}). Green bars represent seedling rootstock families grafted with the CCN51 clonal scion in Cd-spiked soil (7.49 mg kg^{-1}). Data are mean values ± SE (n = 8).

combining abilities were only significantly different for the Cd treatment (**Supplementary Table 3**). Specifically, they differed significantly in 23 of the 38 examined traits, including Cd content in roots, eight nutrient uptake traits, six biomass traits, three cationic interchange traits, and five pigments' content traits (**Table 1**). Meanwhile, only two traits exhibited heritability (h^2) scores above 0.1 under the non-Cd-added treatment, as compared with seven from the Cd-added treatment (**Supplementary Table 4**). Of the latter, all matched with significantly different specific combining abilities (**Table 1**), which speaks for a predominant role of additive genetic effects for ungrafted progenies.

Concerning cacao full-sib seedlings from controlled crosses between IMC67 × PA121 (and reciprocal) 2-months after grafting with the ICS95 and CCN51 scions, specific combining abilities were mostly different under the Cd-added treatment (**Supplementary Table 5**). Specifically, these differences concerned 27 traits from the overall 35 examined traits (**Table 2**). Mismatches due to the scion origin were overserved in 14 cases (including Cd content in leaves and roots). Similarly, when examining the very same cacao seedlings from these controlled crosses 4 months after grafting, specific combining abilities differed for nine of the 17 studied traits (**Supplementary Table 6**). Specifically, these differences only overlapped between the ICS95 and CCN51 clonal scions for Cd content in leaves and dry root weight (**Table 2**).

Most of these specific combining abilities from the full-sib rootstock families fell when looking at the narrow-sense rootstock-mediated heritability (h^2) scores 2 months after grafting (**Supplementary Table 7**), and completely vanished after 4 months (**Supplementary Table 8**). When grafting the clonal scion ICS95, heritability scores above 0.1 were only observed 2 months after grafting for root weight, dry root weight, and protein content, whereas for the CCN51 scion this only applied for chlorophyll *b* (**Table 3**). Four-months after grafting, none h^2 estimate was above 0.1.

DISCUSSION

Cadmium Toxicity Affects a Wide Spectrum of Growth and Physiological Traits Before and After Grafting

In this study, an inhibition in plant growth and biomass was observed as result of Cd toxicity. Cd showed a greater toxic effect on the growth of PA121 OP seedlings than in the other families used as rootstocks. Also, a significant decrease in root biomass

TABLE 1 | Specific combining abilities and narrow-sense heritability (h^2) in cacao seedlings from controlled crosses before grafting.

Trait		MLM						h^2			
		F	*p*	IMC67	PA121	IMC67 × PA121	PA121 × IMC67	Median	2.5%	97.5%	*r*
Cadmium	Cd (mg kg^{-1})	1.80	0.174	85.20	–6.71	2.05	18.54	0.048	0.016	0.183	0.163
	Cd in root (mg kg^{-1})	3.35	**0.035**	40.36	11.59	–0.39	19.48	0.054	0.017	0.231	0.287
Nutrients	K (%)	5.56	**0.005**	1.57	0.15	–0.09	–0.01	0.068	0.022	0.316	0.410
	Mg (%)	5.33	**0.006**	0.48	–0.08	–0.04	–0.08	0.084	0.023	0.351	0.472
	Fe (mg kg^{-1})	17.05	**0.000**	175.50	–40.75	–3.63	7.00	**0.169**	0.034	0.660	0.651
	Mn (mg kg^{-1})	5.32	**0.006**	489.13	79.75	70.75	136.25	0.067	0.021	0.274	0.398
	Cu (mg kg^{-1})	4.16	**0.016**	10.31	1.37	–0.90	–0.26	0.057	0.017	0.233	0.308
	Zn (mg kg^{-1})	5.64	**0.004**	68.01	–15.61	–8.98	–3.56	0.090	0.024	0.350	0.504
	B (mg kg^{-1})	4.03	**0.018**	67.78	–19.04	–10.90	–14.35	0.088	0.023	0.327	0.505
	Na (mg kg^{-1})	8.09	**0.001**	177.63	–30.00	–26.88	–10.50	0.074	0.022	0.303	0.439
Biomass	Leaf fresh weight (g)	7.53	**0.001**	46.55	1.55	11.01	4.78	0.050	0.017	0.332	0.543
	Stem fresh weight (g)	16.53	**0.000**	34.95	–11.38	0.68	–0.61	**0.225**	0.046	0.816	0.705
	Aerial length (cm)	3.89	**0.021**	56.40	–7.15	–0.05	4.31	0.066	0.018	0.285	0.361
	Leaf area (cm^2)	12.19	**0.000**	3217.31	–47.31	1018.14	488.41	0.057	0.016	0.377	0.601
	Dry stem weight (g)	14.50	**0.000**	9.26	–3.04	0.54	–0.09	**0.201**	0.035	0.695	0.675
	Dry root weight (g)	4.06	**0.018**	11.81	–3.68	0.81	–0.07	0.071	0.021	0.336	0.441
Leaf gas exchange	P_N (μmol CO2 m^{-2} s^{-1})	3.38	**0.034**	5.21	–0.04	1.61	3.01	0.049	0.016	0.190	0.310
	WUE_{INST} (μmol CO_2 mmol H_2O)	4.23	**0.015**	0.90	0.02	0.40	0.51	0.048	0.015	0.201	0.428
Electrolyte leakage	Electrolyte leakage (%)	3.10	**0.045**	23.93	4.17	–1.00	–3.89	0.056	0.018	0.245	0.298
Chloroplast pigments	Chlorophyll *A* (mg/g)	11.53	**0.000**	4.49	–1.62	–1.66	–0.97	**0.172**	0.041	0.648	0.647
	Chlorophyll *B* (mg/g)	11.42	**0.000**	1.65	–0.60	–0.59	–0.27	**0.151**	0.030	0.531	0.631
	Total chlorophyll (*a* + *b*) (mg/g)	11.52	**0.000**	6.14	–2.22	–2.25	–1.23	**0.162**	0.037	0.568	0.644
	Carotenoids (mg/g)	13.52	**0.000**	1.37	–0.48	–0.36	–0.36	**0.252**	0.064	0.686	0.740
Total soluble protein	Protein (mg/g)	5.85	**0.004**	0.57	–0.07	0.17	0.35	0.052	0.016	0.231	0.326

Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross. Only significant (bold and underlined) specific combining abilities under the Cd-added treatment are retained, and only h^2 estimates above 0.1 are highlighted, except for the Cd content in leaves that is kept for reference purposes. For the full compilation refer to ***Supplementary Tables 3, 4*** *for the specific combining abilities and the narrow-sense heritability, respectively. Specific combining abilities are indexed to a general mean (intercept under the first column after the p-value), and the corresponding positive or negative contributions (three last columns). Heritability (h^2) and model fits (R software, R Core Team) estimates were computed using an A pedigree relatedness matrix (****Supplementary Figure 2A****) inputted in a "genetic prediction" additive mixed linear model, according to de los Campos et al. (2009).*

of PA121 OP rootstocks was observed both in combinations with clones ICS95 and CCN51 as scions. An effect of toxic levels of Cd on decreasing plant growth and biomass accumulation has been reported for other plant species such as cotton (Farooq et al., 2016), mustard (Ahmad et al., 2011), peanut (Lu et al., 2013), lettuce (Dias et al., 2012), lupin (Zornoza et al., 2002), and *Rorippa globose* (Wei et al., 2012), but this is the first explicit quantification in cacao scion × rootstocks combinations. Two months after grafting, changes were observed in growth parameters, although these were significant only for root biomass. Interestingly, 4 months after grafting, rootstock families having ICS95 as scion continued exhibiting significant changes only for root biomass, whereas the rootstock families having CCN51 as scion already started presenting significant changes also for growth parameters measured on the aerial part such as leaf area and leaf biomass. Decreased leaf area may be the result of reduction in cells size or alternatively more condense intercellular spaces (Barceló et al., 1988). Such effect of Cd on plant growth may result from alterations in cell division (Liu et al., 2003).

Meanwhile, a reduced Cd translocation from root to shoot could be explained by a differential loading of Cd into the xylem (Arao et al., 2010). Differences in Cd uptake by a symplastic pathway could also be related to differences between genotypes in Cd translocation to the stem of rootstocks and to scion's tissues (Arao et al., 2010). The latter would explain a lower accumulation of Cd in ICS95, grafted on IMC67, compared with CCN51 grafted on the same rootstock. Expression of specific transporter proteins could also explain differences in Cd uptake and accumulation in different cacao genotypes (Moore et al., 2020). Still, further studies are needed to evaluate Cd translocation in the xylem of rootstocks × scion combinations to increase knowledge of Cd distribution in plant organs.

TABLE 2 | Specific combining abilities in cacao seedling rootstocks from controlled crosses 2 and 4 months after grafting with the ICS95 and CCN51 scions.

Sampling	Trait		ICS95						CCN51					
			F	*p*	IMC67	PA121	IMC67 × PA121	PA121 × IMC67	*F*	*p*	IMC67	PA121	IMC67 × PA121	PA121 × IMC67
Two months after grafting	Cadmium	Cd in leaf tissue (mg kg^{-1})	5.38	**0.006**	**81.39**	**14.29**	**19.40**	**29.58**	0.55	0.653	121.13	−17.61	−5.95	−3.44
		Cd in roots (mg kg^{-1})	0.26	0.856	33.65	0.76	−1.54	1.44	4.23	**0.016**	**37.37**	**−3.42**	**1.33**	**11.54**
	Nutrients	N (%)	5.31	**0.006**	**1.54**	**0.05**	**−0.04**	**0.27**	6.33	**0.002**	**1.67**	**0.06**	**−0.15**	**0.24**
		K (%)	5.69	**0.004**	**1.76**	**0.31**	**0.08**	**0.02**	6.55	**0.002**	**1.72**	**0.19**	**0.25**	**0.14**
		Mg (%)	3.17	**0.042**	**0.71**	**−0.11**	**−0.01**	**−0.09**	6.59	**0.002**	**0.75**	**−0.13**	**0.01**	**−0.17**
		Cu (mg kg^{-1})	3.04	**0.048**	**8.12**	**3.73**	**1.11**	**2.33**	6.87	**0.002**	**9.98**	**3.19**	**−1.61**	**2.40**
		Zn (mg kg^{-1})	3.74	**0.024**	**63.73**	**−14.80**	**4.51**	**8.70**	2.07	0.130	66.45	−13.83	0.04	−0.30
		Na (mg kg^{-1})	3.18	**0.041**	**113.10**	**12.53**	**21.28**	**−5.64**	2.66	0.070	107.49	−5.00	22.50	2.49
	Biomass	Root fresh weight (g)	9.12	**0.000**	**39.68**	**−12.74**	**1.51**	**1.33**	5.23	**0.006**	**36.39**	**−10.38**	**2.60**	**−0.59**
		Dry root weight (g)	11.08	**0.000**	**16.65**	**−5.88**	**1.16**	**−0.16**	4.71	**0.010**	**15.53**	**−4.58**	**1.05**	**−0.84**
	Chlorophyll fluorescence	Fm	3.03	**0.048**	**377.56**	**−7.56**	**−33.63**	**22.75**	1.51	0.238	370.50	−4.81	−12.38	29.88
		Fv/Fm	4.50	**0.012**	**0.77**	**−0.02**	**−0.02**	**0.01**	1.74	0.185	0.76	−0.03	−0.01	0.01
		Fv/Fo	4.93	**0.008**	**3.41**	**−0.32**	**−0.26**	**0.20**	1.34	0.284	3.11	−0.28	−0.09	0.19
	Chloroplast pigment	Chlorophyll *A* (mg/g)	4.01	**0.019**	**1.53**	**0.03**	**−0.35**	**0.25**	5.80	**0.004**	**1.44**	**0.11**	**−0.44**	**0.09**
		Chlorophyll *B* (mg/g)	4.44	**0.012**	**0.59**	**0.10**	**−0.11**	**0.12**	11.05	**0.000**	**0.64**	**0.58**	**−0.04**	**−0.04**
		Total chlorophyll (*a* + *b*) (mg/g)	4.13	**0.017**	**2.12**	**0.12**	**−0.46**	**0.37**	7.31	**0.001**	**2.08**	**0.69**	**−0.48**	**0.05**
		Carotenoids (mg/g)	5.31	**0.006**	**0.64**	**−0.04**	**−0.14**	**0.10**	4.41	**0.013**	**0.63**	**0.11**	**−0.13**	**0.03**
	Total soluble protein	Protein (mg/g)	13.87	**0.000**	**0.46**	**0.45**	**0.13**	**0.22**	7.04	**0.001**	**0.52**	**0.26**	**0.20**	**0.37**
Four months after grafting	Cadmium	Cd in leaf tissue (mg kg^{-1})	3.96	**0.021**	**66.97**	**6.70**	**16.73**	**40.48**	4.94	**0.009**	**84.10**	**−5.19**	**33.07**	**35.24**
		Cd in roots (mg kg^{-1})	7.08	**0.002**	**35.45**	**21.24**	**−4.21**	**21.30**	0.33	0.803	50.55	−5.33	−8.64	−7.79
	Biomass	Leaf fresh weight (g)	1.76	0.183	69.38	−7.30	−3.18	3.45	7.65	**0.001**	**61.61**	**−2.03**	**−8.83**	**11.96**
		Root weight (g)	3.98	**0.020**	**41.34**	**−4.64**	**8.91**	**7.70**	2.30	0.102	46.11	−7.96	5.16	0.24
		Leaf area (cm^2)	1.34	0.286	4022.02	−324.64	−74.60	293.20	3.50	**0.030**	**3925.93**	**−100.12**	**−121.21**	**843.41**
		Dry leaf weight (g)	2.37	0.097	20.85	−2.95	0.55	2.54	7.74	**0.001**	**18.22**	**0.28**	**−1.66**	**5.19**
		Dry root weight (g)	5.48	**0.005**	**14.68**	**−2.00**	**4.89**	**3.51**	4.93	**0.008**	**16.61**	**−2.56**	**6.30**	**0.60**
	Chlorophyll fluorescence	Fv/Fm	3.30	**0.038**	**0.78**	**−0.01**	**−0.02**	**0.01**	1.06	0.383	0.77	−0.05	0.01	−0.02
		Fv/Fo	3.19	**0.043**	**3.59**	**−0.14**	**−0.35**	**0.29**	1.37	0.275	3.46	−0.34	0.20	−0.31

Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions. Only significant (bold and underlined) specific combining abilities under the Cd-added treatment are retained. For the full compilation refer to ***Supplementary Tables 5, 6*** *for specific combining abilities 2 and 4 months after grafting, respectively. Specific combining abilities are indexed to a general mean (intercept under the first column after the p-value), and the corresponding positive or negative contributions (three last columns).*

TABLE 3 | Narrow-sense rootstock-mediated heritability (h^2) in cacao seedling rootstocks from controlled crosses 2 months after grafting with the ICS95 and CCN51 scions.

Trait		ICS95				CCN51			
		Median	**2.5%**	**97.5%**	***r***	**Median**	**2.5%**	**97.5%**	***r***
Cadmium	Cd in leaf tissue (mg kg^{-1})	0.056	0.018	0.232	0.289	0.052	0.017	0.209	0.200
	Cd in roots (mg kg^{-1})	0.049	0.015	0.196	0.058	0.052	0.017	0.236	0.231
Biomass	Root fresh weight (g)	**0.125**	**0.028**	**0.580**	**0.584**	0.079	0.020	0.325	0.446
	Dry root weight (g)	**0.146**	**0.033**	**0.660**	**0.612**	0.068	0.019	0.289	0.406
Chloroplast pigment	Chlorophyll B (mg/g)	0.054	0.018	0.237	0.267	**0.149**	**0.032**	**0.706**	**0.631**
Total soluble protein	Protein (mg/g)	**0.274**	**0.077**	**0.660**	**0.760**	0.079	0.022	0.362	0.475

Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions. Only h^2 estimates above 0.1 (highlighted) under the Cd-added treatment are retained, except for the Cd content in leaves and roots that is kept for reference purposes (for the full compilation refer to ***Supplementary Tables 7, 8*** *for 4-months after grafting). Heritability (h^2) and model fits (r) estimates were computed using an A pedigree relatedness matrix (****Supplementary Figure 2B****) inputted in a "genetic prediction" additive mixed linear model according to de los Campos et al. (2009).*

Cadmium has an effect on different photosynthetic processes such as decrease in chlorophyll content, inhibition of chlorophyll formation, inhibition of Rubisco activity, inhibition of both photosynthesis reaction centers, PSI and PSII, and an increase in lipoxygenase activity (Rai et al., 2016). The inhibition of the growth of plants subjected to Cd stress could further be due to a toxic effect of the heavy metal on physiological processes, such as reduction of the maximum photochemical efficiency of PSII (Ge et al., 2015; Pereira de Araújo et al., 2017), in addition to an imbalance in the uptake of essential mineral elements (Zornoza et al., 2002; Hédiji et al., 2015) and to an inhibition in the production of sugars due to the decrease in the carbon assimilation rate, P_N (Dias et al., 2012). Ahmad et al. (2011) correlated the toxic effect of Cd on mustard plant growth with a Cd-induced decrease in P_N and WUE. Similarly, a greater decreasing effect of Cd concentration in P_N and WUE_{INST} was observed in our study for PA121 that, as indicated above, was the rootstock that presented a greater effect of Cd toxicity on plant growth. A decrease in WUE suggests alterations in water balance, which could be due to changes in water uptake and transport as a result of the toxic effect of Cd (Singh and Tewari, 2003).

Cadmium binds to several sites in PSII, affecting both the donor and the acceptor side. Cd also inhibits oxygen evolution in a high affinity site by competition with Ca on the donor side (Sigfridsson et al., 2004). Concerning underlying physiological processes, efficiency of PSII, measured as the fluorescence F_v/F_m ratio, did not appear to be affected in Cd-stressed rootstocks. However, significant changes in F_v/F_m ratio of scion ICS95 were observed at 2 and 4 months after grafting on IMC67 × PA121 full-sib family rootstocks established in Cd-spiked soil compared with the control. Interestingly, no significant differences were observed in the maximum efficiency of the reaction centers of the PSII of scion CCN51 grafted on any of the rootstocks subjected to Cd stress in soil. This result suggested that grafting combinations having CCN51 as scion had a more stable photosynthetic performance than those having ICS95 clone as scion. A similar effect of Cd on photosynthetic performance has also been reported in poplar (Ge et al., 2015; Jiao et al., 2015), pea (Sandalio et al., 2001), and lettuce (Dias et al., 2012). One of the factors that could result in an effect on photosynthetic performance is the decrease in chlorophyll content as a result of Cd toxicity (Sandalio et al., 2001). However, for the case of ungrafted rootstocks, an increase in the content of chloroplast pigments at IMC67 OP seedlings is observed and does not result in significant changes in the F_v/F_m ratio. On the other hand, changes in the photosynthetic performance of scion ICS95 2 months after grafting effectively coincided with a decrease in chlorophyll content. In the case of scion CCN51, although a decrease in chlorophyll content is observed under Cd stress, the photosynthetic performance remains unchanged.

Similarly, a significant decrease in the concentration of chloroplast pigments has been reported for other species such as cotton (Farooq et al., 2016), beans (Saidi et al., 2013), *Phragmites australis* (Pietrini et al., 2003), poplar (Jiao et al., 2015), maize (Ekmekçi et al., 2008), pea (Sandalio et al., 2001), and cucumber plants exposed to Cd (Zhang et al., 2002). In this regard, carotenoids play a role as antioxidants, and increases in carotenoid content in some species have been explained as an attempt to protect chlorophyll from the photooxidative damage caused by Cd stress (Mishra et al., 2006). Therefore, the decrease in carotenoid content observed in grafting combinations having ICS95 as scion may have jeopardized the detoxification of radicals formed in response to Cd stress (Ekmekçi et al., 2008).

Cadmium absorption by plants can also result in changes in the accumulation of essential mineral elements in plant tissues (Castro et al., 2015; Pereira de Araújo et al., 2017). Cd may affect the transport of mineral elements by disturbing the radial movement of transporters in the root, loading into the xylem vessels or into the leaves, thereby promoting morphological changes of the xylem tissue, changes in H^+-ATPase activity, and alterations in IRT1 transporter selectivity (Sandalio et al., 2001). Changes in nutritional elements may indicate alterations in ionic homeostasis (Saidi et al., 2013). In our work, changes in mineral elements were observed in rootstock families before grafting, and in both ICS95 and CCN51 leaf tissue 2 months after grafting. Cd translocation in the plant is carried out using the same transporters than some nutritional elements such as Ca, Mn, and Zn (Rahman et al., 2016). Competition for these same transporters may explain the decrease in the content of Zn and Mn in ungrafted rootstocks under Cd stress.

On the other hand, according to Küpper et al. (1998), Cd could replace Mg in chlorophylls, which may decrease chlorophyll content in plants under Cd stress. A direct proportional relationship between toxicity of heavy metals and Mg substitution in chlorophylls has been reported (Küpper et al., 1996). In our study, a significant increase in Mg concentration in leaf tissue was observed 2 months after grafting in Cd-spiked soil. These results are in agreement with the observation made by Ciećko et al. (2005), in which the content of Mg in oat increased when it was grown in Cd-contaminated soil. An effect of Cd on electron transport on the reducing side of photosystem I was observed on isolated chloroplasts of maize plants grown in nutrient solution containing the heavy metal. A reduction in electron transport was associated with a decrease in ferredoxin content, which was then correlated with a low Fe concentration, suggesting that Cd induced Fe deficiency (Siedlecka and Baszyński, 1993). In our work, significant changes in Fe content in leaf tissue of ungrafted rootstocks were observed only for IMC67 seedlings. Accordingly, a decrease in F_v/F_m ratio scores was observed in IMC67 seedlings before grafting. A significant decrease in Fe content has also been observed in bean plants (Saidi et al., 2013). On the other hand, a significant increase in Fe content for cacao in grafting combinations having full-sib IMC67 × PA121 progenies as rootstocks was observed too. Despite this, a significant decrease in F_v/F_m was observed in the ICS95 clone when grafted on this rootstock family, whereas no significant changes were observed in this parameter when using CCN51 as scion.

A decrease in the content of total soluble protein was observed in PA121 seedlings before grafting. Significant decreases were also observed in both CCN51 and ICS95 scions 2 months after grafting on the IMC67 OP rootstock family. An effect in total soluble protein content could be explained by Cd-induced protein degradation and an increase in proteolytic activity (Mishra et al., 2006). In some cases, a slight boost in total soluble protein was observed, but it was not significant compared with the control. Slight increases in protein content may be explained by the induction of stress proteins as part of the plant defense system to Cd toxicity (Di Toppi and Gabbrielli, 1999).

Finally, a significant increase in electrolytes leakage was observed in IMC67 and PA121 established in Cd-spiked soil, suggesting a Cd effect on cell membrane integrity. An increase in electrolyte loss has been observed in cucumber (Gonçalves et al., 2007), maize (Ekmekçi et al., 2008), and *Bacopa monnieri* (Mishra et al., 2006). Heavy metals induce an alteration in the lipid composition of thylakoid membranes. Reactive oxygen species (ROS) induced by Cd results in lipid peroxidation, which implies the degradation of polyunsaturated fatty acids of membrane lipids. The later causes distortion of the lipid bilayer and alters membrane ion channels, resulting in leakage of ions (Mishra et al., 2006). The apparent lack of cell membrane damages in the CCN51 and ICS95 scions, 2 months after grafting, may be due to an insufficient exposure time to Cd as to cause a perceptible effect.

Rootstock Versus Scion Genetic Conflict

This work has enlightened some major trends regarding the complexity at the rootstock–scion interface. First, phenotypic differences due to rootstock effects are more notorious early after grafting (i.e., 2-months), in concert with expectations observed at the ungrafted seedlings. Such differences tend to vanish at older grafted seedlings (i.e., 4 months after grafting). The tendencies observed when computing specific combining abilities across rootstock families are aligned with this conclusion, in the sense that significantly different specific combining abilities among rootstock families were more common at 2 months after grafting than at 4 months. Second, heritability estimates also speak for the dilution of the rootstock effects through genotypes and time. Several of the heritabilities that were calculated in ungrafted families were not significant in the grafted portion of the experiment, which may speak for a genuine physiological communication gap at the graft interface. Alternatively, it could reflect an intrinsic limitation in degrees of freedom due to the complexity of the rootstock × scion factorial design. Still, rootstock-mediated heritability scores above 0.1 were more commonly observed at 2 months after grafting than at 4 months, regardless of the specific rootstock × scion combination. Such instability ultimately suggests an underlying and unavoidable conflict between the two genomes that shape the chimeric grafted organism (Warschefsky et al., 2016; Gautier et al., 2019).

Genetic conflict is known to be pervasive at multiple nested evolutionary scales, for example, among genes, among chromosomes, between chromosomes and cytoplasmic organelles, and between sexes in dioicous species (Ågren and Clark, 2018). These multiple scenarios are evidenced by the recurrent segregation distortion due to gene drives, transposons, and unconventional sex determination systems (Renner and Müller, 2021). However, our study is the first in suggesting an analogous mechanism at the rootstock × scion interface, typically regarded as leading to emergent heterotic properties (Reyes-Herrera et al., 2020). This conflict may be due to underlying additive and combined physiological drivers (Loupit and Cookson, 2020), such as water and nutrients uptake and transport, hormone production and transport, and large-scale movement of molecules during grafting and through time (Rasool et al., 2020).

Foreseeing the Complexity of the Rootstock × Scion Interaction

The novelty of this work lays on the explicit comparison of rootstock families in terms of Cd uptake. However, after quantifying an additive component such as the inheritance of rootstock effects (i.e., rootstock-mediated genetic variance) across recombinant cacao saplings, a next step is to consider more thoroughly the complexity of the rootstock × scion interaction. After all, rootstock metabolites transcend the root system and could reach the grafted scion, i.e., rootstock's additive contribution (Loupit and Cookson, 2020; Rasool et al., 2020), which in turn may have contrasting consequences on rootstock traits, i.e., scion's additive contribution (Shu et al., 2017). These concurrent effects would ultimately feedback an emergent rootstock × scion interaction, a statistical interaction in the strict sense.

To be able to accurately estimate the rootstock × scion component, it will be necessary to further validate the

rootstock-mediated pedigree-estimated heritability scores *via* controlled experiments across an expanded panel of clonal scion genotypes grafted on rootstocks with a more continuous gradient of pairwise relatedness values (Reyes-Herrera et al., 2020). Specifically, future experimental assessments might rely on factorial designs of diverse clonal scions grafted on clonal rootstocks. This would allow to reduce recombination uncertainty and optimize statistical power to estimate the interaction term.

From an analytical point of view, the *A*-BLUP model implemented here would be capable to condition pedigree-based rootstock-mediated heritability scores as a function of the scion's pedigree. Alternatively, the rootstock × scion interaction may also be quantified *via* indirect genetic effect (IGE) models (Bijma, 2010, 2013; Fisher and Mcadam, 2019) at relatively low phenotyping costs. It would also be desirable to extend IGE monitoring through time as a way to validate whether some of the significant rootstock × scion effects may persist in adult trees, even several years after grafting.

Perspectives

A recurrent caveat of pedigree-based heritability estimates concerns the potential fortuitous unbalanced between each phenotypic vector and the *A* pedigree-based relatedness matrix within the "genetic prediction" model. However, we did not detect this trend for any of the significantly rootstock-inherited traits. On the contrary, after examining for 2 years, as part of a parallel experiment, eight traits in full-sib families obtained by cross pollination of clonal accessions from the Cacao Germplasm at greenhouse conditions and two water regimes, we have been able to identify three candidate families for further testing. We recommend using this promissory dataset as reference (i.e., training) population to calibrate explicit eco-physiological mechanistic models (López-Hernández and Cortés, 2019) and last-generation machine learning algorithms (Cortés and López-Hernández, 2021; Montesinos-Loìpez et al., 2021) as innovative alternatives beyond *A*-BLUP models (Guevara-Escudero et al., 2021). As part of this task, we envision the following pipeline: (1) developing explicit eco-physiological indices for cacao targeting neo-tropical localities, (2) calibrating last-generation predictive breeding models aiming to forecast such indices based on extensive genealogical information, (3) extending the previous models to account for the complexity of the rootstock-scion interaction (i.e., G × S × E term, as expanded in the previous section), and (4) validating the corresponding predictions across seed orchards and cacao saplings at local nurseries to leverage natural variation for early selection (i.e., before grafting) of low Cd uptake. Ultimately, this combined strategy promises speeding up breeding of polygenic trait variation in a perennial tree crop, while accounting for the interaction of multiple genotypes at the rootstock–scion interface. In parallel, high throughput genotyping of the rootstock families will enable a more accurate description of the underlying genetic architecture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CR-M conceived the original sampling and experiment and compiled the datasets. JF-P, CH-V, and CR-M collected phenotypic data at the greenhouses. JF-P and CH-V carried out lab work procedures. CH-V, JF-P, and AC prepared input datasets for statistical softwares and carried out data analyses. CR-M and AC drafted a first version of this manuscript and edited by the other co-authors. CH-V, JF-P, AC, MM-D-T, CR-M, and VB interpreted results, contributed to the manuscript, and approved the submitted version.

FUNDING

This research was funded by USAID/USDA Cacao for Peace initiative (Collaboration agreement No. 1939, project 1001136). JF-P received financial support to carry out her master's studies from AGROSAVIA, which is financed with public resources from the Ministry of Agriculture and Rural Development from Colombia (MADR). AGROSAVIA also financed this publication.

ACKNOWLEDGMENTS

We would like to thank the National Germplasm Banks (SBGNAA) for supplying plant material of the cacao genotypes included in this study. We also would like to acknowledge technical support from Diego Fernando Morales Villegas AGROSAVIA, Palmira. We extend our thank to Marshall Elson for commenting an early version of this manuscript, as well as to the reviewers and the editor for their insightful recommendations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.777842/full#supplementary-material

Supplementary Figure 1 | Pearson correlations coefficients among examined traits from half-sib and full-sib seedling rootstocks **(A)** before grafting, **(B)** two, and **(C)** four months after grafting. Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib rootstock progenies obtained from the crossing between IMC67 × PA12 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions under two Cd treatments. Correlation estimates are presented above the diagonal and below diagonal circles are sized and coloured accordingly.

Supplementary Figure 2 | Heat plots of A pedigree relatedness matrices from two OP half-sib families (IMC67 and PA121), and two full-sib rootstock families derived from controlled crosses between IMC67 × PA121 and its reciprocal cross, inputed in a "genetic prediction" additive mixed linear model, according to de los Campos et al. (2009). Heat plots are depicted for verification purposes **(A)** before

grafting, and **(B)** two and **(C)** four months after grafting with the ICS95 and CCN51 clonal scions under two Cd treatments.

Supplementary Table 1 | Concentration of mineral nutrients in leaf tissue of ungrafted rootstocks five months after cadmium treatment. Different lowercase letters indicate significant differences in mineral elements concentration between ungrafted seedling families at the same Cd concentration in soil. Different capital letters indicate significant differences in mineral elements concentration of the same ungrafted seedling at different Cd concentrations in soil. Results are according to Duncan test ($p < 0.05$). Data are mean values ± SE ($n = 8$).

Supplementary Table 2 | Concentration of mineral nutrients in leaf tissue of scions ICS95 and CCN51 two months after grafting onto rootstocks. Different lowercase letters indicate significant differences in mineral elements concentration in leaf tissue of the ICS95 or CCN5 clonal scions grafted on four different seedling rootstock families at the same Cd concentration in soil. Different capital letters indicate significant differences in mineral elements concentration in leaf tissue of ICS95 or CCN51 clonal scions grafted on the same seedling rootstock family at different Cd concentrations in soil. Results are according to Duncan test ($p < 0.05$). Data are mean values ± SE ($n = 8$).

Supplementary Table 3 | Specific combining abilities in cacao seedlings from controlled crosses before grafting. Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, under two Cd treatments. Specific combining abilities are indexed to a general mean (intercept under the first column after the *p*-value), and the corresponding positive or negative contributions (three last columns). Significantly different specific combining abilities are highlighted.

Supplementary Table 4 | Narrow-sense heritability (h2) in cacao seedlings from controlled crosses before grafting. Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions under two Cd treatments. Heritability (h^2) and model fits (r) estimates were computed using an *A* pedigree relatedness matrix (**Supplementary Figure 2A**) inputted in a "genetic prediction" additive mixed linear model, according to de los Campos et al. (2009). Heritability (h^2) scores above 0.1 are highlighted.

Supplementary Table 5 | Specific combining abilities in cacao seedling rootstocks from controlled crosses two-months after grafting. Estimates were gathered in full-sib rootstock progenies obtained from the crossing between IMC67 × PA121, and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions under two Cd treatments. Specific combining abilities are indexed to a general mean (intercept under the first column after the *p*-value), and the corresponding positive or negative contributions (three last columns). Significantly different specific combining abilities are highlighted.

Supplementary Table 6 | Specific combining abilities in cacao seedling rootstocks from controlled crosses four-months after grafting. Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions under two Cd treatments. Specific combining abilities are indexed to a general mean (intercept under the first column after the *p*-value), and the corresponding positive or negative contributions (three last columns). Significantly different specific combining abilities are highlighted.

Supplementary Table 7 | Narrow-sense rootstock-mediated heritability (h2) in cacao seedling rootstocks from controlled-crosses two-months after grafting. Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions under two Cd treatments. Heritability (h^2) and model fits (r) estimates were computed using an *A* pedigree relatedness matrix (**Supplementary Figure 2B**) inputted in a "genetic prediction" additive mixed linear model, according to de los Campos et al. (2009). Heritability (h^2) scores above 0.1 are highlighted.

Supplementary Table 8 | Narrow-sense rootstock-mediated heritability (h2) in cacao seedling rootstocks from controlled-crosses four-months after grafting. Estimates were gathered in two OP half-sib families (IMC67 and PA121), and two full-sib progenies obtained from the crossing between IMC67 × PA121 and its reciprocal cross, grafted with the ICS95 and CCN51 clonal scions under two Cd treatments. Heritability (h^2) and model fits (r) estimates were computed using an *A* pedigree relatedness matrix (**Supplementary Figure 2C**) inputted in a "genetic prediction" additive mixed linear model, according to de los Campos et al. (2009). None h^2 estimate was above 0.1.

REFERENCES

Ågren, J. A., and Clark, A. G. (2018). Selfish genetic elements. *PLoS Genet.* 14:e1007700. doi: 10.1371/journal.pgen.1007700

Ahmad, P., Nabi, G., and Ashraf, M. (2011). Cadmium-induced oxidative damage in mustard [*Brassica juncea* (L.) Czern. & Coss.] plants can be alleviated by salicylic acid. *South Afr. J. Bot.* 77, 36–44. doi: 10.1016/j.sajb.2010.05.003

Arao, T., Ishikawa, S., Murakami, M., Abe, K., Maejima, Y., and Makino, T. (2010). Heavy metal contamination of agricultural soil and counter measures in Japan. *Paddy and water Environ.* 8, 247–257. doi: 10.1007/s10333-010-0205-7

Arao, T., Takeda, H., and Nishihara, E. (2008). Reduction of cadmium translocation from roots to shoots in eggplant (*Solanum melongena*) by grafting onto *Solanum torvum* rootstock. *Soil Sci. Plant Nutr.* 54, 555–559. doi: 10.1111/j.1747-0765.2008.00269.x

Arenas, S., Cortés, A. J., Mastretta, A., and Jaramillo, J. P. (2021). Evaluating the accuracy of genomic prediction for the management and conservation of relictual natural tree populations. *Tree Genet. Genomes* 17, 1–19. doi: 10.1007/s11295-020-01489-1

Arévalo-Gardini, E., Arévalo-Hernández, C. O., Baligar, V. C., and He, Z. L. (2017). Heavy metal accumulation in leaves and beans of cacao (*Theobroma cacao* L.) in major cacao growing regions in Peru. *Sci. Total Environ.* 605, 792–800. doi: 10.1016/j.scitotenv.2017.06.122

Argüello, D., Chavez, E., Lauryssen, F., Vanderschueren, R., Smolders, E., and Montalvo, D. (2019). Soil properties and agronomic factors affecting cadmium concentrations in cacao beans: a nationwide survey in Ecuador. *Sci. Total Environ.* 649, 120–127. doi: 10.1016/j.scitotenv.2018.08.292

Asman, A., bin Purung, M. H., Lambert, S., Amiruddin, A., and Rosmana, A. (2021). Effect of rootstock and scion on resistance of cocoa clones to vascular streak dieback caused by *Ceratobasidium theobromae*. *Ann. Agricult. Sci.* 66, 25–30. doi: 10.1016/j.aoas.2021.02.005

Barceló, J., Vázquez, M. D., and Poschenrieder, C. H. (1988). Structural and ultrastructural disorders in cadmium-treated bush bean plants (*Phaseolus vulgaris* L.). *New Phytol.* 108, 37–49. doi: 10.1111/j.1469-81371988.tb00202.x

Barraza, F., Schreck, E., Lévêque, T., Uzu, G., López, F., Ruales, J., et al. (2017). Cadmium bioaccumulation and gastric bioaccessibility in cacao: a field study in areas impacted by oil activities in Ecuador. *Environ. Pollut.* 229, 950–963. doi: 10.1016/j.envpol.2017.07.080

Bijma, P. (2010). Estimating indirect genetic effects: precision of estimates and optimum designs. *Genetics* 186, 1013–1028. doi: 10.1534/genetics.110.120493

Bijma, P. (2013). The quantitative genetics of indirect genetic effects: a selective review of modelling issues. *Heredity* 112, 61–69. doi: 10.1038/hdy.2013.15

Borek, M., Bączek-Kwinta, R., and Rapacz, M. (2013). "Chlorophyll fluorescence imaging of cadmium-treated white cabbage plants," in *Proceedings of the 16th International Conference on Heavy Metals in the Environment E3S Web of Conferences*, Vol. 1, (Les Ulis: EDP Sciences), 39004.

Boughalleb, N., Tarchoun, N., El-Mbarki, A., and El-Mahjoub, M. (2007). Resistance evaluation of nine cucurbit rootstocks and grafted watermelon (*Citrullus lanatus* L.) varieties against *Fusarium* wilt and *Fusarium* crown and root rot. *J. Plant Sci.* 2, 102–107. doi: 10.3923/jps.2007.102.107

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Bradstreet, R. B. (1954). Kjeldahl method for organic nitrogen. *Anal. Chem.* 26, 185–187.

Cardinal, A. B. B., Gonçalves, P. D. S., and Martins, A. L. M. (2007). Stock-scion interactions on growth and rubber yield of *Hevea brasiliensis*. *Sci. Agric.* 64, 235–240. doi: 10.1590/S0103-90162007000300004

Castro, A. V., de Almeida, A. A. F., Pirovani, C. P., Reis, G. S., Almeida, N. M., and Mangabeira, P. A. (2015). Morphological, biochemical, molecular and ultrastructural changes induced by Cd toxicity in seedlings of *Theobroma cacao* L. *Ecotoxicol. Environ. Safety* 115, 174–186. doi: 10.1016/j.ecoenv.2015.02.003

Chavez, E., He, Z. L., Stoffella, P. J., Mylavarapu, R. S., Li, Y. C., Moyano, B., et al. (2015). Concentration of cadmium in cacao beans and its relationship with soil cadmium in southern Ecuador. *Sci. Total Environ.* 533, 205–214. doi: 10.1016/j.scitotenv.2015.06.106

Chen, Z. Q., Baison, J., Pan, J., Karlsson, B., Andersson, B., Westin, J., et al. (2018). Accuracy of genomic selection for growth and wood quality traits in two control-pollinated progeny trials using exome capture as the genotyping platform in Norway spruce. *BMC Genomics* 19:946.

Ciećko, Z., Kalembasa, S., Wyszkowski, M., and Rolka, E. (2005). The magnesium content in plants in soil contaminated with cadmium. *Polish J. Environ. Stud.* 14, 365–370.

Cortés, A. J., and López-Hernández, F. (2021). Harnessing crop wild diversity for climate adaptation. *Genes* 12:783.

Cuevas, J., Crossa, J., Soberanis, V., Pèirez-Elizalde, S., Pèirez-Rodrüguez, P., de los Campos, G., et al. (2016). Genomic prediction with genotype x environment interaction kernel regres- sion models. *Plant Genome* 9, 1–20.

Davis, A. R., and Perkins-Veazie, P. (2005). Rootstock effects on plant vigour and watermelon fruit quality. *Cucurbit Genet. Coop. Rep.* 28, 39–42.

de los Campos, G., Naya, H., Gianola, D., Crossa, J., Legarra, A., Manfredi, E., et al. (2009). Predicting quantitative traits with regression models for dense molecular markers and pedigree. *Genetics* 182, 375–385. doi: 10.1534/genetics.109.101501

de los Campos, G., Sorensen, D., and Gianola, D. (2015).). Genomic heritability: what is it? *PLoS Genet.* 11:e1005048. doi: 10.1371/journal.pgen.1005048

Di Toppi, L. S., and Gabbrielli, R. (1999). Response to cadmium in higher plants. *Environ. Exp. Bot.* 41, 105–130. doi: 10.1016/S0098-8472(98)00058-6

Dias, M. C., Monteiro, C., Moutinho-Pereira, J., Correia, C., Gonçalves, B., and Santos, C. (2012). Cadmium toxicity affects photosynthesis and plant growth at different levels. *Acta Physiol. Plant.* 35, 1281–1289. doi: 10.1007/s11738-012-1167-8

Ekmekçi, Y., Tanyolac, D., and Ayhan, B. (2008). Effects of cadmium on antioxidant enzyme and photosynthetic activities in leaves of two maize cultivars. *J. Plant Physiol.* 165, 600–611. doi: 10.1016/j.jplph.2007.01.017

Eltayb, M. T. A., Magid, T. D. A., Ibrahim, A. A., and Dirar, A. M. A. (2014). Effect of grafting (rootstock) on morphological changes of scions in some Acacia species. *J. Forest Prod. Ind. Corpus.* 3, 27–36.

Engbersen, N., Gramlich, A., Lopez, M., Schwarz, G., Hattendorf, B., Gutierrez, O., et al. (2019). Cadmium accumulation and allocation in different cacao cultivars. *Sci. Total Environ.* 678, 660–670. doi: 10.1016/j.scitotenv.2019.05.001

European Commission (2014). Commission Regulation (EC) No 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs. *Official J. Eur. Union* 138:75.

Farooq, M. A., Ali, S., Hameed, A., Bharwana, S. A., Rizwan, M., Ishaque, W., et al. (2016). Cadmium stress in cotton seedlings: physiological, photosynthesis and oxidative damages alleviated by glycinebetaine. *South Afr. J. Bot.* 104, 61–68. doi: 10.1016/j.sajb.2015.11.006

Fisher, D. N., and Mcadam, A. G. (2019). Indirect genetic e ects clarify how traits can evolve even when fitness does not. *Evol. Lett.* 3, 4–14. doi: 10.1002/evl3.98

Gautier, A. T., Chambaud, C., Brocard, L., Ollat, N., Gambetta, G. A., Delrot, S., et al. (2019). Merging genotypes: graft union formation and scion-rootstock interactions. *J. Exp. Bot.* 70, 747–755. doi: 10.1093/jxb/ery422

Ge, W., Jiao, Y., Zou, J., Jiang, W., and Liu, D. (2015). Ultrastructural and photosynthetic response of populus 107 leaves to cadmium stress. *Polish J. Environ. Stud.* 24, 519–527. doi: 10.15244/pjoes/27814

Guevara-Escudero, M., Osorio, A. N., and Cortés, A. J. (2021). Integrative breeding for biotic resistance in forest trees. *Plants* 10:2022.

Gonçalves, J. F., Becker, A. G., Cargnelutti, D., Tabaldi, L. A., Pereira, L. B., Battisti, V., et al. (2007). Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings. *Braz. J. Plant Physiol.* 19, 223–232. doi: 10.1590/S1677-04202007000300006

Gutierrez, A. P., Matika, O., Bean, T. P., and Houston, R. D. (2018). Genomic selec- tion for growth traits in Pacific oyster (*Crassostrea gigas*): potential of low-density marker panels for breeding value prediction. *Front. Genet.* 9:391. doi: 10.3389/fgene.2018.00391

Hédiji, H., Djebali, W., Belkadhi, A., Cabasson, C., Moing, A., Rolin, D., et al. (2015). Impact of long-term cadmium exposure on mineral content of *Solanum lycopersicum* plants: consequences on fruit production. *South Afr. J. Bot.* 97, 176–181. doi: 10.1016/j.sajb.2015.01.010

Isaac, R. A., and Kerber, J. D. (1971). "Atomic absorption and flame photometry: techniques and uses in soil, plant, and water analysis," in *Instrumental Methods for Analysis of Soils and Plant Tissue*, ed L. M. Walsh. doi: 10.2136/1971.instrumentalmethods.c2

Isele, E., Breen, M., and Galanti, R. (2020). *Grafting Cacao. College of Tropical Agriculture and Human Resources (CTAHR)*.

Jiao, Y., Ge, W., Qin, R., Sun, B., Jiang, W., and Liu, D. (2012). Influence of cadmium stress on growth, ultrastructure and antioxidative enzymes in Populus 2025. *Fresenius Environ. Bull.* 21, 1375–1384.

Jiao, Y., Zou, J., Ge, W., Jiang, W., and Liu, D. (2015). Physiological and ultrastructural effects of cadmium on Poplar (Populus x euramericana) leaves. *Baltic Forestry* 21, 106–113.

Küpper, H., Kupper, F., and Spiller, M. (1996). Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants. *J. Exp. Bot.* 47, 259–266. doi: 10.1093/jxb/47.2.259

Küpper, H., Küpper, F., and Spiller, M. (1998). In situ detection of heavy metal substituted chlorophylls in water plants. *Photosynth. Res.* 58, 123–133.

Lewis, C., Lennon, A. M., Eudoxie, G., and Umaharan, P. (2018). Genetic variation in bioaccumulation and partitioning of cadmium in *Theobroma cacao* L. *Sci. Total Environ.* 640, 696–703. doi: 10.1016/j.scitotenv.2018.05.365

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* 148, 350–382.

Liu, D., Jiang, W., and Gao, X. (2003). Effects of cadmium on root growth, cell division and nucleoli in root tip cells of garlic. *Biol. Plant.* 47, 79–83. doi: 10.1023/A:1027384932338

Liu, S., Li, H., Lv, X., Ahammed, G. J., Xia, X., Zhou, J., et al. (2016). Grafting cucumber onto luffa improves drought tolerance by increasing ABA biosynthesis and sensitivity. *Sci. Rep.* 6, 1–14. doi: 10.1038/srep20212

López-Hernández, F., and Cortés, A. J. (2019). Last-generation genome–environment associations reveal the genetic basis of heat tolerance in common bean (*Phaseolus vulgaris* L.). *Front. Genet.* 10:954. doi: 10.3389/fgene.2019.00954

Loupit, G., and Cookson, S. J. (2020). Identifying molecular markers of successful graft union formation and compatibility. *Front. Plant Sci.* 11:352. doi: 10.3389/fpls.2020.610352

Lu, Z., Zhang, Z., Su, Y., Liu, C., and Shi, G. (2013). Cultivar variation in morphological response of peanut roots to cadmium stress and its relation to cadmium accumulation. *Ecotoxicol. Environ. Safety* 91, 147–155. doi: 10.1016/j.ecoenv.2013.01.017

Lux, A., Martinka, M., Vaculík, M., and White, P. J. (2011). Root responses to cadmium in the rhizosphere: a review. *J. Exp. Bot.* 62, 21–37. doi: 10.1093/jxb/erq281

Melgarejo, L. M., Romero, M., Hernández, S., Jaime, S. M. E., Suárez, D., and Pérez, W. (2010). *Experimentos en Fisiología Vegetal Lab Físiol Bioquímica Veg.* Bogotá: Universidad Nacional de Colombia. Available online at: https://www.uv.mx/personal/tcarmona/files/2019/02/Melgarejo-2010.pdf

Mishra, S., Srivastava, S., Tripathi, R. D., Govindarajan, R., Kuriakose, S. V., and Prasad, M. N. V. (2006). Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L?. *Plant Physiol. Biochem.* 44, 25–37. doi: 10.1016/j.plaphy.2006.01.007

Montesinos-Loìpez, O. A., Montesinos-Loìpez, A., Peìrez-Rodriìguez, P., Barroìn-Loìpez, J. A., Martini, J. W. R., and Fajardo-Flores, S. B. (2021). A review of deep learning applications for genomic selection. *BMC Genomics* 22:19.

Moore, R. E., Ullah, I., de Oliveira, V. H., Hammond, S. J., Strekopytov, S., Tibbett, M., et al. (2020). Cadmium isotope fractionation reveals genetic variation in Cd uptake and translocation by *Theobroma cacao* and role of natural resistance-associated macrophage protein 5 and heavy metal ATPase-family transporters. *Horticult. Res.* 7, 1–11. doi: 10.1038/s41438-020-0292-6

Müller, B. S. F., Neves, L. G., de Almeida Filho, J. E., Resende, M. F. R., Munþoz, P. R., et al. (2017). Genomic prediction in contrast to a genome-wide association study in explaining heritable variation of complex growth traits in breeding populations of Eucalyptus. *BMC Genomics* 18:1–17. doi: 10.1186/s12864-017-3920-2

Nguyen, N. T., McInturf, S. A., and Mendoza-Cózatl, D. G. (2016). Hydroponics: a versatile system to study nutrient allocation and plant responses to nutrient availability and exposure to toxic elements. *J. Visual. Exp. JoVE* 113:54317. doi: 10.3791/54317

Palencia Calderon, G. E., Gómez Santos, R., and Mejia, L. A. (2007). *Patrones Para Cacao: Cartilla (No. Doc. 22050) CO-BAC, Bogotá)*. Bucaramanga: Editorial AGROSAVIA.

Pereira de Araújo, R., Furtado, de Almeida, A. A., Pereira, L. S., Mangabeira, P. A., Souza, J. O., et al. (2017). Photosynthetic, antioxidative, molecular and ultrastructural responses of young cacao plants to Cd toxicity in the soil. *Ecotoxicol. Environ. Safety* 144, 148–157. doi: 10.1016/j.ecoenv.2017.06.006

Pérez-Rodríguez, P., and de los Campos, G. (2014). Genome-wide regression and prediction with the Bglr statistical package. *Genetics* 198, 483–495. doi: 10.1534/genetics 114.164442

Pietrini, F., Iannelli, M. A., Pasqualini, S., and Massacci, A. (2003). Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplasts of *Phragmites australis* (Cav.) Trin. ex Steudel. *Plant Physiol.* 133, 829–837. doi: 10.1104/pp.103.026518

Rahman, A., Mostofa, M. G., Nahar, K., Hasanuzzaman, M., and Fujita, M. (2016). Exogenous calcium alleviates cadmium-induced oxidative stress in rice (Oryza sativa L.) seedlings by regulating the antioxidant defense and glyoxalase systems. *Braz. J. Bot.* 39, 393–407. doi: 10.1016/j.crvi.2016.08.002

Rai, R., Agrawal, M., and Agrawal, S. B. (2016). "Impact of heavy metals on physiological processes of plants: with special reference to photosynthetic system," in *Plant Responses to Xenobiotics*, (Singapore: Springer), 127–140. doi: 10.1007/978-981-10-2860-1_6

Rasool, A., Mansoor, S., Bhat, K. M., Hassan, G. I., Baba, T. R., Alyemeni, M. N., et al. (2020). Mechanisms underlying graft union formation and rootstock scion interaction in horticultural plants. *Front. Plant Sci.* 11:590847. doi: 10.3389/fpls.2020.590847

Renner, S. S., and Müller, N. A. (2021). Plant sex chromosomes defy evolutionary models of expanding. *Nat. Plants* 7, 392–402. doi: 10.1038/s41477-021-00884-3

Reyes-Herrera, P. H., Muñoz-Baena, L., Velásquez-Zapata, V., Patiño, L., Delgado-Paz, O. A., Díaz-Diez, C. A., et al. (2020). Inheritance of rootstock effects in avocado (*Persea americana* Mill.) cv. *Hass. Front. Plant Sci.* 11:555071. doi: 10.3389/fpls.2020.555071

Ribeiro, M. A., de Almeida, A. A. F., Alves, T. F., Gramacho, K. P., Pirovani, C. P., and Valle, R. R. (2016). Rootstock× scion interactions on *Theobroma cacao* resistance to witches' broom: photosynthetic, nutritional and antioxidant metabolism responses. *Acta Physiol. Plant.* 38:73. doi: 10.1007/s11738-016-2095-9

Ríos, A. V. (1957). La relación patrón-injerto en la propagación vegetativa del cacao (*Theobroma cacao* L.). *Acta Agron.* 7, 223–232.

Rivard, C. L., and Louws, F. J. (2008). Grafting to manage soilborne diseases in heirloom tomato production. *HortScience* 43, 2104–2111. doi: 10.21273/HORTSCI.43.7.2104

Rouphael, Y., Cardarelli, M., Rea, E., and Colla, G. (2008). Grafting of cucumber as a means to minimize copper toxicity. *Environ. Exp. Bot.* 63, 49–58. doi: 10.1016/j.envexpbot.2007.10.015

Saidi, I., Ayouni, M., Dhieb, A., Chtourou, Y., Chaïbi, W., and Djebali, W. (2013). Oxidative damages induced by short-term exposure to cadmium in bean plants: protective role of salicylic acid. *South Afr. J. Bot.* 85, 32–38. doi: 10.1016/j.sajb.2012.12.002

Sandalio, L. M., Dalurzo, H. C., Gomez, M., Romero-Puertas, M. C., and Del Rio, L. A. (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp. Bot.* 52, 2115–2126. doi: 10.1093/jexbot/52.364.2115

Savvas, D., Colla, G., Rouphael, Y., and Schwarz, D. (2010). Amelioration of heavy metal and nutrient stress in fruit vegetables by grafting. *Sci. Horticult.* 127, 156–161. doi: 10.1016/j.scienta.2010.09.011

Shu, B., Liu, L., Jue, D., Wang, Y., Wei, Y., and Shi, S. (2017). Effects of avocado (*Persea americana* Mill.) scion on arbuscular mycorrhizal and root hair development in rootstock. *Arch. Agron. Soil Sci.* 63, 1951–1962.

Siedlecka, A., and Baszyński, T. (1993). Inhibition of electron flow around photosystem I in chloroplasts of Cd-treated maize plants is due to Cd-induced iron deficiency. *Physiol. Plant.* 87, 199–202.

Sigfridsson, K. G., Bernát, G., Mamedov, F., and Styring, S. (2004). Molecular interference of Cd2+ with Photosystem II. *Biochim. Biophys. Acta (BBA)-Bioenergetics* 1659, 19–31. doi: 10.1016/j.bbabio.2004.07.003

Singh, P. K., and Tewari, R. K. (2003). Cadmium toxicity induced changes in plant water relations and oxidative metabolism of *Brassica juncea* L. plants. *J. Environ. Biol.* 1, 107–112.

Song, Y., Jin, L., and Wang, X. (2017). Cadmium absorption and transportation pathways in plants. *Int. J. Phytoremed.* 19, 133–141. doi: 10.1080/15226514.2016.1207598

Spanò, R., Ferrara, M., Gallitelli, D., and Mascia, T. (2020). The role of grafting in the resistance of tomato to viruses. *Plants* 9:1042. doi: 10.3390/plants9081042

Suárez-Hernández, ÁM., Vázquez-Angulo, J. C., Grimaldo-Juárez, O., Duran, C. C., González-Mendoza, D., Bazante-González, I., et al. (2019). Production and quality of grafted watermelon in saline soil. *Horticult. Brasil.* 37, 215–220. doi: 10.1590/S0102-053620190213

Sugiyama, M., Ae, N., and Arao, T. (2007). Role of roots in differences in seed cadmium concentration among soybean cultivars—proof by grafting experiment. *Plant Soil* 295, 1–11. doi: 10.1007/s11104-007-9252-2

Ullah, I., Wang, Y., Eide, D. J., and Dunwell, J. M. (2018). Evolution, and functional analysis of natural resistance-associated macrophage proteins (NRAMPs) from *Theobroma cacao* and their role in cadmium accumulation. *Sci. Rep.* 8, 1–15. doi: 10.1038/s41598-018-32819-y

Vanderschueren, R., Montalvo, D., De Ketelaere, B., Delcour, J. A., and Smolders, E. (2019). The elemental composition of chocolates is related to cacao content and origin: a multi-element fingerprinting analysis of single origin chocolates. *J. Food Composition Anal.* 83:103277. doi: 10.1016/j.jfca.2019.103277

Vanderschueren, R., Argüello, D., Blommaert, H., Montalvo, D., Barraza, F., Maurice, L., et al. (2021). Mitigating the level of cadmium in cacao products: reviewing the transfer of cadmium from soil to chocolate bar. *Sci. Total Environ.* 146779. doi: 10.1016/j.scitotenv.2021.146779

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., Von Wettberg, E. J. B., et al. (2016). Rootstocks: diversity, domestication, and impacts on shoot phenotypes. *Trends Plant Sci.* 21, 418–437. doi: 10.1016/j.tplants.2015.11.008

Wei, S., Li, Y., Zhan, J., Wang, S., and Zhu, J. (2012). Tolerant mechanisms of *Rorippa globosa* (Turcz.) Thell. hyperaccumulating Cd explored from root morphology. *Bioresource Technol.* 118, 455–459. doi: 10.1016/j.biortech.2012.05.049

Yanyan, Y. A. N., Shuoshuo, W. A. N. G., Min, W. E. I., Biao, G. O. N. G., and Qinghua, S. H. I. (2018). Effect of different rootstocks on the salt stress tolerance in watermelon seedlings. *Horticult. Plant J.* 4, 239–249. doi: 10.1016/j.hpj.2018.08.003

Yin, J. P. T. (2004). Rootstock effects on cocoa in Sabah, Malaysia. *Exp. Agricult.* 40, 445–452. doi: 10.1017/S0014479704002108

Zhang, F., Shi, W., Jin, Z., and Shen, Z. (2002). Response of antioxidative enzymes in cucumber chloroplasts to cadmium toxicity. *J. Plant Nutr.* 26, 1779–1788. doi: 10.1081/PLN-120023282

Zhang, H., Yin, L., Wang, M., Yuan, X., and Liu, X. (2019). Factors affecting the accuracy of genomic selection for agricultural economic traits in maize, cattle, and pig populations. *Front. Genet.* 10:189. doi: 10.3389/fgene.2019.00189

Zornoza, P., Vázquez, S., Esteban, E., Fernández-Pascual, M., and Carpena, R. (2002). Cadmium-stress in nodulated white lupin: strategies to avoid toxicity. *Plant Physiol. Biochem.* 40, 1003–1009. doi: 10.1016/S0981-9428(02)01464-X

Author Disclaimer: The findings and conclusion in this publication are those of the authors and should not be construed to represent any official USDA or United States Government determination or policy.

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 May 2022
doi: 10.3389/fpls.2022.843099

Inheritance of Yield Components and Morphological Traits in Avocado cv. Hass From "Criollo" "Elite Trees" *via* Half-Sib Seedling Rootstocks

Gloria Patricia Cañas-Gutiérrez[1,2], Stella Sepulveda-Ortega[1†], Felipe López-Hernández[1], Alejandro A. Navas-Arboleda[1‡] and Andrés J. Cortés[1*†‡]*

[1] Corporación Colombiana de Investigación Agropecuaria AGROSAVIA, C.I. La Selva, Rionegro, Colombia, [2] Corporation for Biological Research (CIB), Unit of Phytosanity and Biological Control, Medellín, Colombia

Grafting induces precocity and maintains clonal integrity in fruit tree crops. However, the complex rootstock × scion interaction often precludes understanding how the tree phenotype is shaped, limiting the potential to select optimum rootstocks. Therefore, it is necessary to assess (1) how seedling progenies inherit trait variation from elite 'plus trees', and (2) whether such family superiority may be transferred after grafting to the clonal scion. To bridge this gap, we quantified additive genetic parameters (i.e., narrow sense heritability—h^2, and genetic-estimated breeding values—GEBVs) across landraces, "criollo", "plus trees" of the super-food fruit tree crop avocado (*Persea americana* Mill.), and their open-pollinated (OP) half-sib seedling families. Specifically, we used a genomic best linear unbiased prediction (*G*-BLUP) model to merge phenotypic characterization of 17 morpho-agronomic traits with genetic screening of 13 highly polymorphic SSR markers in a diverse panel of 104 avocado "criollo" "plus trees." Estimated additive genetic parameters were validated at a 5-year-old common garden trial (i.e., provenance test), in which 22 OP half-sib seedlings from 82 elite "plus trees" served as rootstocks for the cv. Hass clone. Heritability (h^2) scores in the "criollo" "plus trees" ranged from 0.28 to 0.51. The highest h^2 values were observed for ribbed petiole and adaxial veins with 0.47 (CI 95%0.2–0.8) and 0.51 (CI 0.2–0.8), respectively. The h^2 scores for the agronomic traits ranged from 0.34 (CI 0.2–0.6) to 0.39 (CI 0.2–0.6) for seed weight, fruit weight, and total volume, respectively. When inspecting yield variation across 5-year-old grafted avocado cv. Hass trees with elite OP half-sib seedling rootstocks, the traits total number of fruits and fruits' weight, respectively, exhibited h^2 scores of 0.36 (± 0.23) and 0.11 (± 0.09). Our results indicate that elite "criollo" "plus trees" may serve as promissory donors of seedling rootstocks for avocado cv. Hass orchards due to the inheritance of their outstanding trait values. This reinforces the feasibility to leverage natural variation from "plus trees" *via* OP half-sib seedling rootstock families. By jointly estimating half-sib family effects and rootstock-mediated heritability, this study promises boosting seedling rootstock breeding programs, while better discerning the consequences of grafting in fruit tree crops.

Keywords: ***Persea americana*** **Mill., heritability, rootstock effects, seed-mediated grafting, "criollo" avocado tree**

OPEN ACCESS

Edited by:
Vittorino Novello, University of Turin, Italy

Reviewed by:
Bode A. Olukolu, The University of Tennessee, Knoxville, United States
Joaquin Guillermo Ramirez Gil, National University of Colombia, Colombia

****Correspondence:***
Gloria Patricia Cañas-Gutiérrez
gcanas@cib.org.co
Andrés J. Cortés
acortes@agrosavia.co

†Secondary addresses:
Stella Sepulveda-Ortega, Facultad de Ciencias Agrarias, Politécnico Colombiano Jaime Icaza Cadavid, Medellín, Colombia

Andrés J. Cortés, Facultad de Ciencias Agrarias—Departamento de Ciencias Forestales, Universidad Nacional de Colombia—Sede Medellín, Medellín, Colombia

‡These authors share senior authorship

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *24 December 2021*
Accepted: *10 February 2022*
Published: *24 May 2022*

Citation:
Cañas-Gutiérrez GP, Sepulveda-Ortega S, López-Hernández F, Navas-Arboleda AA and Cortés AJ (2022) Inheritance of Yield Components and Morphological Traits in Avocado cv. Hass From "Criollo" "Elite Trees" via Half-Sib Seedling Rootstocks. Front. Plant Sci. 13:843099. doi: 10.3389/fpls.2022.843099

INTRODUCTION

Grafting is an ancient technique used for vegetative propagation, especially in perennial fruit crops. This method, used on woody and herbaceous plants, can improve several agronomic characteristics, such as yield and vigor, as well as tolerance to biotic and abiotic stresses (Loupit and Cookson, 2020). In fruit trees, grafting is a common propagation method because it provides a dual plant system to increase orchard productivity by maintaining genetic uniformity of the commercial clones. Usually, scions' buds with high quality are grafted onto rootstocks with improved stress/disease tolerance. In this dual plant system, rootstocks are also selected to increase orchard efficiency through vigor control and yield improvement of the scion. Increasing precocity is another benefit of grafting, as scions taken from mature trees show significantly earlier bearing and maturity relative to trees grown from seeds. Therefore, many tree crops with long juvenile phases are grown as grafted plants to obtain a faster return on investment (Ahsan et al., 2019). Despite these benefits, grafting tends to obscure individual genotypic contributions from the scion and the rootstock to the overall tree phenotype, which makes genetic selection harder at particular rootstock × scion combinations.

From a physiological perspective, such confounding factors are pervasive, too. Broadly speaking, grafting affects three main processes at the tree level: uptake and transport of water and nutrients (Little et al., 2016), production and transport of hormones, and large-scale movement of proteins, messenger RNAs, and small RNAs (Wang et al., 2017; Loupit and Cookson, 2020; Lu et al., 2020; Rasool et al., 2020). These processes have implications for both subsurface and surface functioning, yet the interconnection of variables at the rootstock—scion interface still hides contributions from the individual genotypes (Tworkoski and Miller, 2007; Amiri et al., 2014; Warschefsky et al., 2016). After all, both rootstock and scion genotypes play an important role at the grafting interface, and different rootstock × scion combinations mutually alter their individual phenotypic effects (Goldschmidt, 2014). Additional factors that may affect the rootstock × scion interactions are the age of the bud-donor tree, the grafting technique, seasonality, time since grafting, the genotype × environment interaction (Albacete et al., 2015), the rootstock × scion compatibility, and microbiome-root interactions (Warschefsky et al., 2016).

A highly valued grafted clonal super-food fruit tree crop that is rapidly expanding around the world is avocado (*Persea americana* Mill.) cv. Hass (O'Brien et al., 2018). Improved rootstocks for commercial avocado cv. Hass plantations may confer a beneficial horticultural quality to the tree across a wide spectrum of traits (Reyes-Herrera et al., 2020), such as increased fruit yield (Herrera-González et al., 2013), postharvest performance (Willingham et al., 2001), vegetative vigor (Mickelbart and Arpaia, 2002), salt tolerance (Bernstein et al., 2001), and disease resistance (Smith et al., 2011; Sánchez-González et al., 2019). These reports are in line with previous research that have shown how rootstocks might also induce less trivial scion morphological changes, such as dwarfing, and even alter yield traits and fruit quality (Egea et al., 2004; Picolotto et al., 2010; Madam et al., 2011; Expósito et al., 2020; Kviklys and Samuoliene, 2020). For instance, rootstock effects may even influence properties typically attributed to the clonal Hass scion, such as fruit sensorial and nutritional quality, e.g., texture, sugar content, acidity, pH, flavor, and color (Giorgi et al., 2005; Gullo et al., 2014; Balducci et al., 2019), cold tolerance, and shoot pest and pathogen resistance (Rubio et al., 2005; Goldschmidt, 2014).

Most commercial avocado Hass plantations in Neotropical areas currently rely on open-pollinated (OP) half-sib interracial seedling rootstocks derived from selected "criollo" "plus trees" (Bernal et al., 2014; Cañas-Gutiérrez et al., 2015). Therefore, avocado production is mainly based on grafting the commercial Hass cultivar onto untested highly diverse seedling "criollo" saplings (Rodríguez et al., 2009; Cañas-Gutiérrez et al., 2015). As part of this procedure, the selection of a suitable rootstock is rarely based on both the genotype of the scion, and the environment or agro-climatic zone in which the grafted tree will be cultivated. In other words, due to a triple rootstock × scion × environment interaction, rootstock selection from "criollo" seedling genotypes is challenging. Still, growing half-sib families of seedling rootstocks from selected donor "plus trees" remains a promising strategy because they may harbor natural adaptations to the highly heterogeneous ecosystems found at the Neotropics, not to mention the fact that they constitute an important source of genetic variability (Kuhn et al., 2019; Rendón-Anaya et al., 2019; Talavera et al., 2019; López-Guzmán et al., 2021) for otherwise clonal Hass plantations (Cañas-Gutiérrez et al., 2019a).

Avocado rootstock breeding programs typically perform an initial selection step for resistance to soil-borne pathogens, such as *Phytophthora cinnamomi* Rans. Yield and adaptive traits are then left as second and third steps within avocado rootstock breeding. To guarantee that selected rootstocks exhibit a stable phenotypic effect, avocado nurseries in temperate latitudes often rely on clonal rootstock propagation (Téliz, 2000). However, clonal rootstocks are still difficult to produce because they require double grafting techniques (Ernst, 1999), while *in vitro* production has not been commercially scaled (Hormaza, 2020). Therefore, clonal rootstocks are still rare in highly diverse Neotropical regions where avocados are native, and seedling saplings from "criollo" trees are more abundant and cheaper. Because of this, it is imperative to assess which traits of the avocado "criollo" are inherited to its OP half-sib seedling progenies, while at the same time transferred to the scion after grafting. Specifically, an explicit estimation of combined half-sib families and rootstock heritability effects would be a major advance to speed-up seedling rootstock breeding programs, and ultimately discern the consequences of grafting for tropical avocados (Reyes-Herrera et al., 2020).

To fill this research gap, the goals of this work were to: (1) estimate additive genetic parameters (i.e., narrow sense heritability scores and breeding values) across promissory avocado "criollo" "plus trees," (2) quantify the inheritance of agronomic traits of avocado "criollo" "plus trees" in cv. Hass *via* OP half-sib seeding families, and (3) identify promissory avocado "criollo" "plus trees" as seed donors for rootstock production based on their genetic-estimated breeding values for

heritable morphological and agronomic traits. We hypothesize that heritable variation for key traits would enable half-sib seedling rootstock selection from elite "criollo" "plus trees." Specifically, we have relied on a total of 104 avocado "criollo" trees between 40 and 50 years old, identified as "plus trees" in the province of Antioquia (northwest Colombia). These avocado trees are long-term survivors across different remote agro-ecological zones, so we expect that they may serve as potential donors of seedling rootstocks due to their presumably outstanding adaptability and trait values (e.g., health, longevity, and productivity). These "criollo" avocados had previously been characterized with microsatellite (single sequence repeat, SSR) markers to study their genetic diversity, as well as in terms of their morpho-agronomic traits (Cañas-Gutiérrez et al., 2019a,b). Therefore, this study merged both genetic and phenotypic characterizations to assist seedling rootstock selection at early nursery stages. Estimated additive genetic parameters were validated at a 5-year-old common garden trial (i.e., provenance test) that established OP half-sib seedlings from elite "plus trees" as rootstocks for the Hass clone.

MATERIALS AND METHODS

Plant Material

A total of 104 avocado "criollo" "plus trees" were selected in different remote agroecological zones up to 2,400 m asl of the province of Antioquia, northwest Andes of Colombia, during the years 2014 and 2015 (originally enlisted under the first table of Cañas-Gutiérrez et al. (2019b), and summarized here under **Supplementary Table 1**). Their selection was made based on criteria, such as adaptability to their growing area, longevity, health, and productivity. During this period, trees were *in situ* characterized by taking morphological data, and carrying out their molecular fingerprinting (section below on "microsatellite markers characterization"). Collection of yield data was additionally carried out in these trees during four consecutive years (2014–2017).

The seeds produced by selected elite avocado "criollo" trees were germinated at greenhouses, and obtained 3-month-old seedlings were grafted with cv. Hass scions. In the year 2015, the cv. Hass grafts were planted at a common garden (i.e., provenance test) at AGROSAVIA's research station La Selva, located in Rionegro, province of Antioquia, at 2,100 m asl. Morphological data, such as length and diameter of the rootstock, the canopy length and diameter of the scion, and the morphology of the graft scar were collected during the years 2017–2021. The production data of the cv. Hass grafts were collected in the same period from 2017 to 2021. Since half-sib seedling rootstocks grafted with the Hass clone have been in production during five consecutive years in the same environment (La Selva locality), we were able to minimize environmental variance and equal phenotypic variance to the rootstocks' additive genetic component, which is the main benefit of carrying out common garden or provenance tests. These trials intend to neutralize environmental effects over phenotypic expression, by planting diverse genotypes in a single locality (Lascoux et al., 2016). Our setup has allowed heritability analyses to include a total of 9 years of production data, both for *in situ* "criollo" "plus trees" (2014–2017), as well as in their OP half-sib seedling progenies used as rootstocks for the Hass scion (2017–2021).

Measurements of Morpho-Agronomic Traits

A total of 17 morphological and agronomic traits were measured during the sampling trips of 2014 and 2015 in the avocado "criollo" selected "plus trees," according to descriptors that are specific for avocado based on the International Plant Genetic Resources Institute (IPGRI). These descriptors included tree, leaf, fruit, and seed traits (**Table 1** and **Supplementary Table 2**). Fruit production was recorded in these trees during four consecutive years until 2017. Pearson's, Spearman's and Kendall's correlation matrices (**Figure 1**) were visually inspected using customized plots drawn with the *cor.test* and *heatmap* functions in R v.3.4.4 (R Core Team). Complete phenotypic measures were possible for a total of 82 "criollo" "plus trees" from the original sampling.

Furthermore, a total of 22 OP half-sib seedling progenies of 82 elite avocado "criollo" "plus trees" were used as rootstocks for the Hass scion. Grafted trees were characterized at AGROSAVIA's La Selva research station in terms of their early growth traits (i.e., young shoot color, stem color, stem lenticels, lenticels color, average number of leaves at transplant, and average stem diameter at grafting), as well as in terms of their production (i.e., cumulative total number of fruits and fruit's weight) 5 years after grafting (2017–2021). The analyses focused on yield traits because they allowed for a direct comparison with the recorded production traits in the "criollo" "plus trees," besides the fact that

TABLE 1 | Phenotypic traits measured in the avocado "criollo" selected "plus trees," according to descriptors for avocado of the International Plant Genetic Resources Institute (IPGRI).

Type of trait	Phenotypic traits	Abbreviation	Units of measurement
Morphological traits	Tree shape	TSH	Qualitative
	Trunk circumference	TC	Centimeters
	Trunk surface	TS	Qualitative
	Branching	B	Qualitative
	Leaf texture	LT	Qualitative
	Leaf shape	LS	Qualitative
	Leaf margin	LM	Qualitative
	Tip of leaf shape	TLS	Qualitative
	Number main veins	NMV	Number
	Adaxial Veins	AV	Qualitative
	Ribbed petiole	RP	Qualitative
Agronomic traits	Fruit length	FL	Centimeters
	Fruit diameter	FD	Centimeters
	Fruit weight	FW	Grams
	Seed weight	SW	Grams
	Pulp weight	PW	Grams
	Total volume	TV	Grams

For details on quality measures and their codifications refer to ***Supplementary Table 2****.*

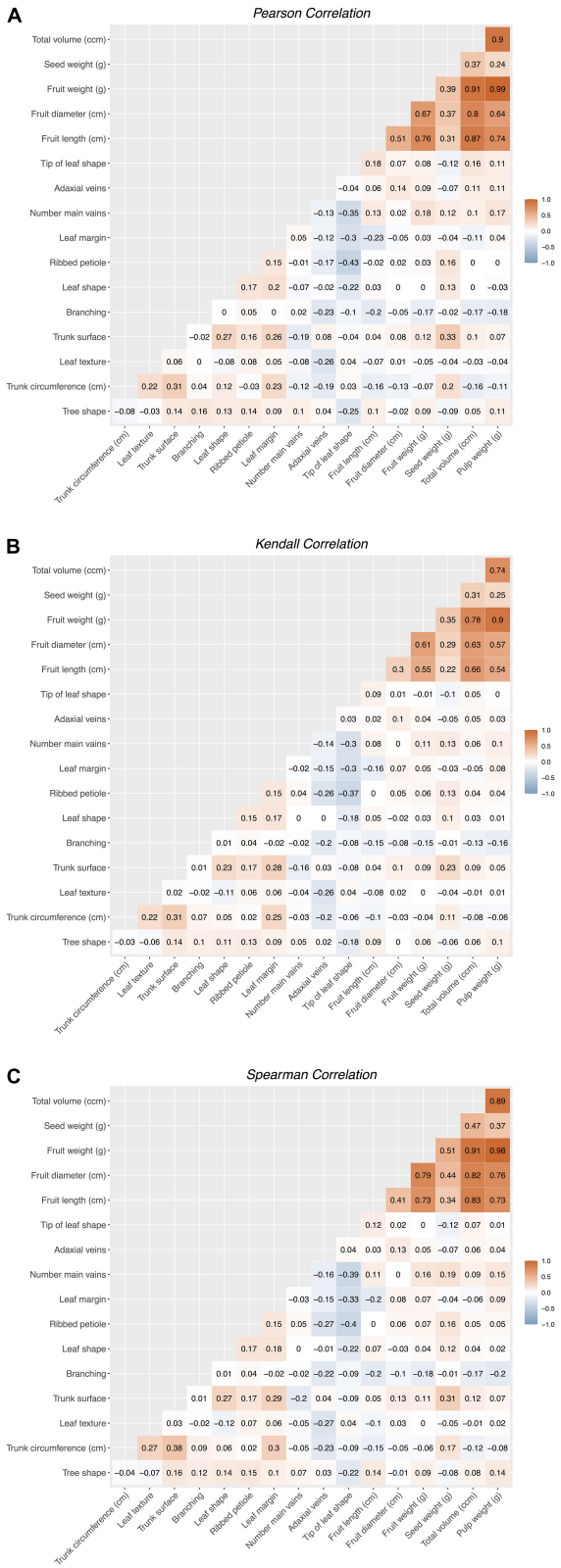

FIGURE 1 | Pearson **(A)**, Spearman **(B)**, and Kendall **(C)** correlation matrices among studied traits in avocado "criollo" "plus trees." For details on the measured traits please refer to **Table 1** and **Supplementary Table 2**.

yield traits are of paramount interest for commercial avocado cv. Hass orchards, especially those aiming for exportation markets.

Microsatellite Marker Characterization

Total genomic DNA was obtained from avocado "criollo" leaves based on the extraction method standardized by Cañas-Gutiérrez et al. (2015). Thirteen microsatellites [simple sequence repeats (SSRs)], originally designed by Sharon et al. (1997) and Ashworth et al. (2004), were chosen (**Supplementary Table 3**) for their high polymorphism information content following estimates by Alcaraz and Hormaza (2007). Fingerprinting highly polymorphic SSR markers allowed disclosing pedigree-free marker-based relationships among genotypes, which in turn enabled distinguishing from highly related to completely unrelated individuals. Such contrasting scales of SSR-reconstructed shared relatedness due to recent co-ancestry (i.e., isolation by descendent, IBD) offer the basis to quantify phenotypic clustering patterns across family types (i.e., genetic heritability), as demonstrated in oil palm (Cros et al., 2015) and rubber tree (Cros et al., 2019), and as expanded in the section below.

Three multiplex PCR amplifications were performed in 10 μl volume containing 16 mM (NH4)2SO4, 67 mM Tris-HCl pH 8.8, 0.01% Tween20, 2 mM MgCl2, 0.1 mM each dNTP,0.4 μM of each primer, 25 ng genomic DNA and GoTaq® ADN polymerase (Promega, WI, United States). Forward primers were labeled with WellRed fluorescent dyes on the 5′ end (Proligo, France). The reactions were carried out in an I-cycler (Bio-Rad Laboratories, Hercules, CA, United States) thermo cycler using the following temperature profile for multiplex PCR 2 and 3: an initial step of 1 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, and a final step of 5 min at 72°C. For multiplex PCR 1, the same temperature profiles were used, except for 55°C for annealing temperature that was used instead. The PCR products were analyzed by capillary electrophoresis in the equipment ABI PRISM® 3130 Genetic Analyzer (Applied Biosystem, CA, United States). Allele sizes were estimated in base pairs with a Peak Scanner (Thermo Fisher Scientific, United States), allowing for a maximum of two alleles per sample.

Computation of Heritability Scores and Breeding Values

We implemented a *G*-BLUP (genomic best linear unbiased predictor) model to quantify genetic parameters (i.e., narrow-sense heritability and breeding values) for key agronomic traits across the avocado "criollo" "plus trees" selected as seed donors for the OP half-sib progenies used as rootstocks for the Hass clone. Genetic parameters are a baseline of any breeding program (Holland et al., 2003) since they guarantee that additive genetic gains are maximized, while breeding cycles are minimized (Dieters et al., 1995). This is because genetic parameters account for the proportion of phenotypic variance among individuals in a population due to genetic effects (Milner et al., 2000; Kruuk, 2004; Berenos et al., 2014). In this sense, the *G*-BLUP precisely offers a modern pedigree-free marker-based approach (Meuwissen et al., 2001; Crossa et al., 2017) to estimate genetic parameters on

populations of mixed ancestry (Frentiu et al., 2008; Wilson et al., 2010), such as OP seedlings.

We first computed a *G* relatedness matrix, following Lynch and Ritland (1999), to account for the half-sib structure of the OP seedling progenies used as rootstocks. To validate relationships within the *G* matrix we further computed (1) a distance tree by means of the Neighbor Joining algorithm (Kamvar et al., 2014) in R (R Core Team) with 10,000 bootstrap replicates, as well as (2) an unsupervised Bayesian clustering using the STRUCTURE software (Pritchard et al., 2000) with five independent runs for each *K*-value from $K = 2$ to $K = 8$ and 100,000 Monte Carlo Markov chain replicates with a burn-in of 50,000.

The admixed origin of the avocado "criollo" genepool, due to avocado's protogynous dichogamy, is known to favor an adequately variable *G* matrix, embracing various degrees of relationship, as much as the Avocado Genebank (Cañas-Gutiérrez et al., 2019b). The variability in the *G* matrix (from highly related to completely unrelated trees) enabled the use of the *G*-BLUP predictor. Hence, we relied on this *G* relationship matrix to compute the genetic-based *G*-BLUP model, following Arenas et al. (2021) and Fernandez-Paz et al. (2021), as in:

$$y = Xb + Za + e$$

where y corresponds to the phenotypic trait vector, a is the vector of individual random additive genetic effects with a normal distribution [in other words, $a \sim N(0, G\,\sigma_a{}^2)$, where G is the among-tree pedigree-free SSR-based relationship matrix, and $\sigma_a{}^2$ corresponds to the additive genetic variance (Müller et al., 2017)], b is the vector of fixed effects (i.e., general mean, intercept), e is the vector of residual effects, and X and Z are the incidence matrices for fixed effects and additive genetic effects (Chen et al., 2018; Gutierrez et al., 2018).

Parameter estimation for the *G*-BLUP model was carried out using reproducing kernel Hilbert space (RKHS) implemented in the BGLR (Bayesian Generalized Linear Regression) software (Perez and De Los Campos, 2014) under an R v.3.4.4 environment (R Core Team). The multi-dimensional space of parameters was sampled with 10,000 iterations, an initial burn-in of 5,000 steps, and a thinning interval of 10 for data recording. Trace plots were drawn in R to verify convergence in the posterior distributions.

Narrow sense rootstock-mediated heritability (h^2), which is the proportion of the overall phenotypic variance accounted for additive genetic variance, was then calculated by retrieving the additive ($\sigma_a{}^2$) and residual ($\sigma_e{}^2$) variances from the RKHS algorithm, following de los Campos et al. (2015):

$$h^2 = \sigma_a^2/(\sigma_a^2 + \sigma_e^2)$$

The vector of h^2 scores was summarized using the median and the 95% CI of the BGLR's posterior distribution. Statistical accuracy (a.k.a., prediction ability, r_y) was computed per trait using Pearson's correlation between observed and predicted (μ + GEBV) trait values (Müller et al., 2017; Zhang et al., 2019). Predicted trait values were recorded and subtracted to the overall phenotypic mean (μ), to gather the vector of genetic-estimated breeding values (GEBVs), which is the deviation from the overall trait mean attributed to additive genetic effects. GEBVs were summarized throughout a biplot diagram using R's *princomp* and *biplot* functions for the principal components analysis (PCA).

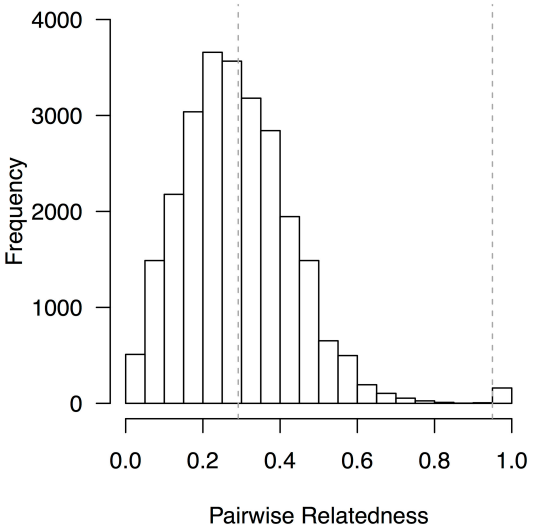

FIGURE 2 | Pairwise relatedness among avocado "criollo" "plus trees" according to Lynch and Ritland (1999). The overall mean and the 0.95 threshold to identify genetic controls are marked with dashed vertical lines.

Finally, we implemented mother-offspring regressions to estimate h^2 scores for key agronomic traits in the OP half-sib seedling progenies of avocado "criollo" "plus trees" grafted with the Hass scion. This heritability analysis included 5 years of data in the OP half-sib seedling progenies of avocado "criollo" "plus trees" used as rootstocks for the Hass scion *via* the mother vs. half-sib regression analysis (Falconer et al., 1996). These h^2 estimates were gathered for cumulative number of fruits and weight of fruits from the Hass clone grafted on top of the OP half-sib seedling rootstocks derived from the selected avocado "criollo" "plus trees." Mother-offspring regressions were carried out in R using the function *lm*. Regressions were plotted for validation in *ggplot*, under the same R environment. The narrow-sense heritability score was calculated as twice the slope from the mother-offspring regressions, following Lynch and Walsh (1998).

RESULTS

Heritability of Morphological and Agronomic Traits in Avocado "Criollo" "Plus Trees"

The 13 microsatellite markers that were used for the molecular characterization of 104 avocado "criollo" "plus trees" amplified a total of 147 allelic fragments. This indicated sufficient polymorphism to reconstruct the pedigree-free marker-based relationship matrix (**Figure 2**), while distinguishing highly related from completely unrelated admixed individuals (**Figure 3**). The phylogenetic tree (**Supplementary Figure 1**) corroborated the latter point. Therefore, the segregation of 147

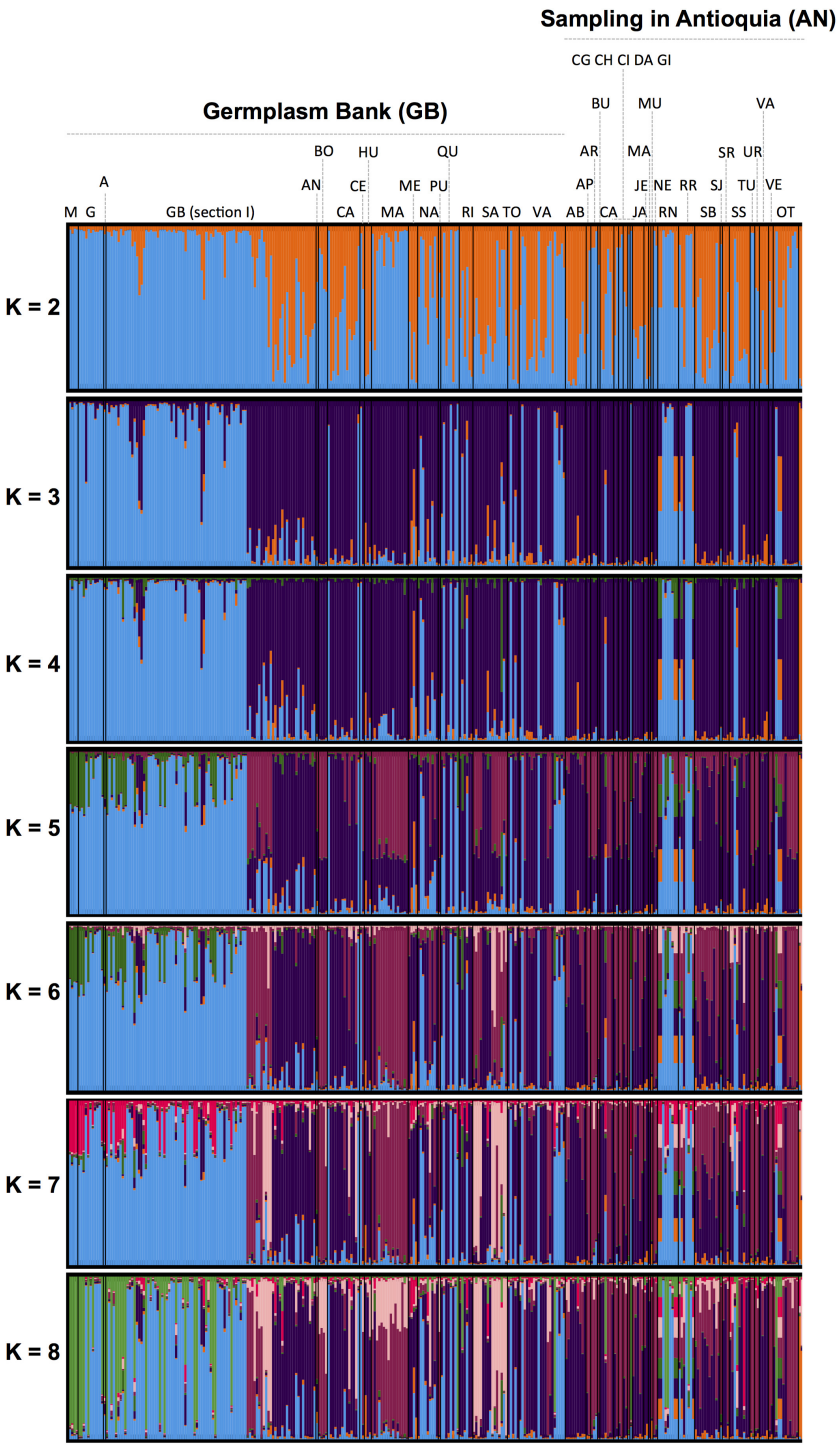

FIGURE 3 | Unsupervised Bayesian clustering among avocado "criollo" "plus trees" collected from the northwest Andes of Colombia (province of Antioquia, AN), and the avocado Germplasm Bank (GB) from Colombia as characterized by Cañas-Gutiérrez et al. (2019a) with the same panel of SSR markers. M, G, and A, stand for Mexican, Guatemalan, and West Indies race controls, respectively. GB Section 1 comprises commercial genotypes. The remaining two-letter abbreviation codes indicate avocado "criollo" trees, respectively sampled from different provinces (under GB) and villages (under AN) across Colombia and the province of Antioquia (northwest Andes of Colombia), as follows: Antioquia (AN), Bolivar (BO), Cauca (CA), Cesar (CE), Huila (HU), Magdalena (MA), Meta (ME), Nariño (NA), Putumayo (PU), Quindío (QU), Risaralda (RI), Santander (SA), Tolima (TO), and Valle del Cauca (VA) for provinces across Colombia, and Abejorral (AB), Apartadó (AP), Arboletes (AR), Buriticá (BU), Caramanta, Carepa or Caracolí (CA), Cañas Gordas (CG), Chigorodó (CH), Cisneros (CI), Dabeiba (DA), Giraldo (GI), Jardín (JA), Jericó (JE), Maceo (MA), Mutatá (MU), Necoclí (NE), Rionegro (RN), Urrao (RR), Santa Bárbara (SB), San Juan (SJ), San Roque (SR), Sonsón (SS), Turbo (TU), Uramita (UR), Valparaiso (VA), Venecia (VE), and other (OT) for municipalities within the province of Antioquia. For further details on the sampling within Antioquia, such as sub-region and elevation, please refer to **Supplementary Table 1** and the first table in Cañas-Gutiérrez et al. (2019b).

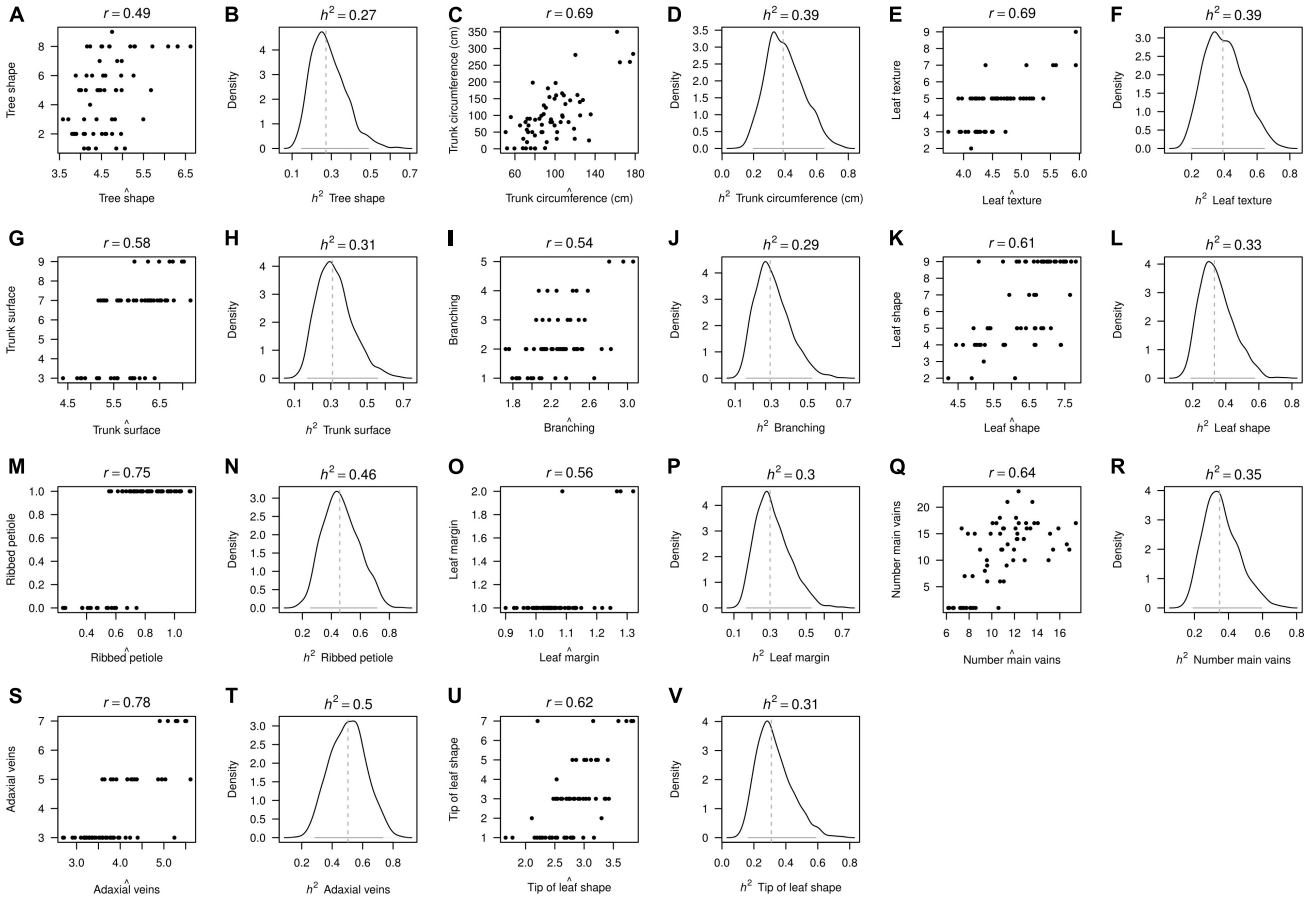

FIGURE 4 | Narrow-sense heritability (h^2) posterior distributions for morphological traits measured in avocado "criollo" "plus trees" selected as seed donors for rootstocks of the cv. Hass. Model fits are depicted in the odd columns **(A,C,E,G,I,K,M,O,Q,S,U)** using Pearson's correlation *r* score between the observed (y-axis) and the predicted (x-axis) trait values. Meanwhile, median values (vertical dashed lines) and the 95% confidence intervals (horizontal gray lines) are depicted in the even columns **(B,D,F,H,J,L,N,P,R,T,V)**. Different traits are sorted every second subpanel across rows according to the x-label in each subfigure.

allelic SSR fragments allowed to effectively quantify phenotypic clustering patterns across family types *via* de *G*-BLUP model (i.e., SSR-based genetic heritability estimates, Cros et al., 2015, 2019) for key morphological and agronomic traits in "criollo" avocado "plus trees."

The heritability parameters for a total of 11 morphological traits (**Figure 4**) and 6 agronomical traits (**Figure 5**) were computed in 82 of the 104 avocado "criollo" "plus trees" selected as seed donors for rootstocks of the cv. Hass. Estimates of avocado "criollo" "plus trees" narrow-sense heritabilities (h^2) were significant for the 11 measured morphological traits and for the 6 agronomic traits, according to the 95% CIs from the posterior distribution, all of which were above absolute zero (**Table 2**).

The estimated heritabilities (h^2) for the 11 morphological traits ranged from 0.28 to 0.51. The tree shape and branching traits presented the h^2 lowest scores, 0.28 and 0.29, respectively. The highest h^2 values were presented for ribbed petiole and adaxial veins with 0.47 and 0.51, respectively. The 6 agronomic traits evaluated presented very similar h^2 values that ranged from 0.34 to 0.39. Seed weight was the trait with the lowest h^2 score (0.34), while fruit weight and total volume traits exhibited the highest values, both with 0.39 (**Table 2**).

Confidence intervals for the heritability scores were generally narrow for the 17 examined traits. The traits that presented the widest confidence intervals were: number main veins (0.1–0.7, r = 0.64) pulp weight (0.1–0.7, r = 0.66), adaxial veins (0.2–0.8, r = 0.78), and ribbed petiole (0.2–0.8, r = 0.75). Meanwhile, the trait that presented the narrowest confidence interval was the shape of the tree with 0.1–0.4 and r = 0.69 (**Table 2**). Adaxial veins and ripped petiole traits exhibited the widest 95% CIs, as well as the highest heritability scores (0.51 and 0.47, respectively). On the contrary, the tree shape trait had the lowest 95% CI, and also showed the lowest heritability score (0.28). Broadly speaking, the morphological and agronomic traits measured in avocado "criollo" "plus trees" selected as seed donors for rootstocks of the cv. Hass presented heritabilities in a medium range. This suggested a relative contribution of additive genetic effects in the

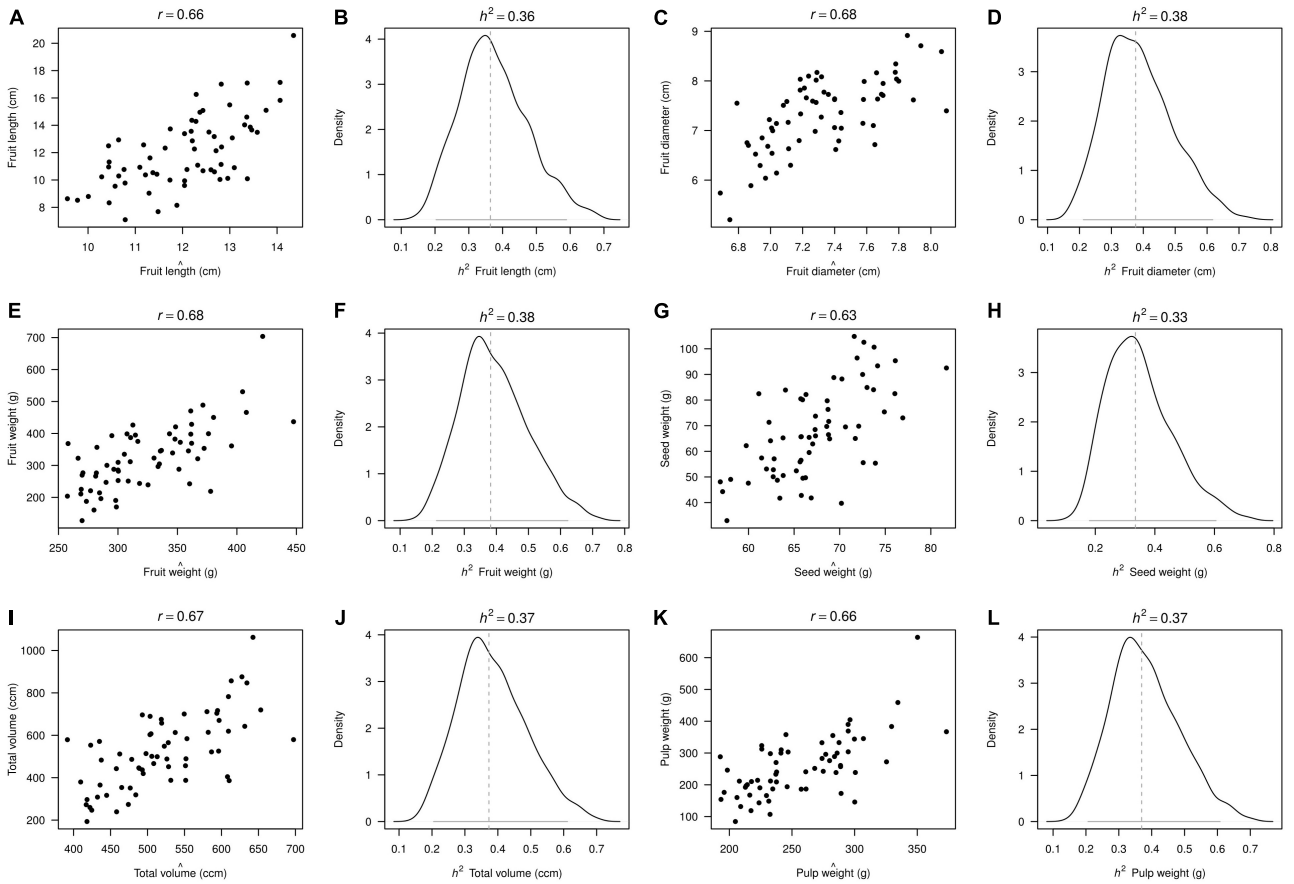

FIGURE 5 | Narrow-sense heritability (h^2) posterior distributions for agronomic traits measured in avocado "criollo" "plus trees" selected as seed donors for rootstocks of the cv. Hass. Model fits are depicted in the odd columns **(A,C,E,G,I,K)** using Pearson's correlation *r* score between the observed (y-axis) and the predicted (x-axis) trait values. Meanwhile, median values (vertical dashed lines) and the 95% confidence intervals (horizontal gray lines) are depicted in the even columns **(B,D,F,H,J,L)**. Different traits are sorted every second subpanel across rows according to the x-label in each subfigure.

total variation of these traits, which may enable moderate genetic gains *via* recurrent selection.

Breeding Values of Avocado "Criollo" "Plus Trees" to Select Target Seed Donors

To assist the ranking of avocado "criollo" "plus trees" as seed donors for OP half-sib seedling rootstocks of the cv. Hass, we summarized the breeding values for the 11 morphological and 6 agronomical traits across 63 of the 104 avocado "criollo" "plus trees" selected as seed donors for rootstocks of the cv. Hass using a biplot diagram from a PCA (**Figure 6**). The length of each vector in the biplot diagram allowed ranking the traits by their additive variance. In this sense, fruit diameter, total volume, fruit weight, pulp weight, fruit length, and branching exhibited the most notorious additive genetic contributions, reinforcing the heritability results described in the previous section. On the contrary, the ribbed petiole and trunk circumference traits, showed lower vector lengths, indicating a lower additive genetic variance for these traits. These results suggested that fruit traits tended to exhibit more potential to respond to selection, indicating that production at commercial avocado cv. Hass orchards may be leveraged by a proper selection of avocado "criollo" "plus trees" as seedling rootstock donors, based on their breeding values.

The biplot diagram also allowed pinpointing the elite avocado "criollo" trees that may be promising for selection given their heritable superiority in terms of agronomical traits. For instance, the trait vector for fruit diameter (associated with the fruits' caliber, an important factor for its commercialization) pointed toward "criollo" trees ANSS9 and ANNE55. Similarly, the trait vectors for total fruits' volume, fruits' length, and fruits' weight, respectively pointed toward the avocado "criollo" "plus trees" encoded as ANDA62, ANCI75, and ANCH65. Meanwhile, the avocado "criollo" tree encoded as ANTU57 was closed to the vectors for the traits fruit diameter and pulp weight, which also makes ANTU57 a promising donor for seedling rootstock families (**Figure 6**). On the other hand, the morphological traits branching, leaf shape, trunk surface, tree shape, and adaxial veins presented the longest vectors, and consequently greater potential for selection. Some of these morphological

TABLE 2 | Narrow—Sense heritability (h^2) estimates for the 17 measured traits from 82 avocado "criollo" "plus trees" selected as seed donors for rootstocks.

	Phenotypic traits	h^2	$IC_{95\%}$	*r*
Morphological traits	Tree shape	0.27	0.15–0.46	0.49
	Trunk circumference (cm)	0.39	0.20–0.64	0.69
	Trunk surface	0.31	0.16–0.53	0.58
	Branching	0.29	0.16–0.54	0.54
	Leaf texture	0.39	0.20–0.60	0.69
	Leaf shape	0.33	0.18–0.55	0.61
	Leaf margin	0.30	0.16–0.51	0.56
	Tip of leaf shape	0.31	0.15–0.58	0.62
	Number main veins	0.35	0.19–0.58	0.64
	Adaxial veins	0.50	0.26–0.72	0.78
	Ribbed petiole	0.46	0.26–0.71	0.75
Agronomic traits	Fruit length (cm)	0.36	0.19–0.58	0.66
	Fruit diameter (cm)	0.38	0.21–0.60	0.68
	Fruit weigh (g)	0.38	0.22–0.60	0.68
	Seed weight (g)	0.33	0.18–0.61	0.63
	Pulp weight (g)	0.37	0.21–0.58	0.66
	Total volume (cm^2)	0.37	0.20–0.59	0.67

Narrow-sense heritability (h^2) and model fits (Pearson's r between the observed and the predicted trait value) estimates were gathered using Lynch and Ritland's (1999) relatedness matrix inputted in a "genetic prediction" additive mixed linear model, according to de los Campos et al. (2009). The significance of heritability scores was determined according to the 95% confidence intervals from posterior distributions, all of which were above the absolute zero.

traits may be of interest for half-sib rootstock family selection. For example, branching and tree shape may contribute desired tree architectures optimum for light penetration to boost flowering and harvesting.

Inheritance of Key Agronomic Traits on the Hass Scion Through Open-Pollinated Half-Sib Seedling Rootstocks of Avocado "Criollo" "Plus Trees"

A total of 22 "criollo" "plus trees" were selected as seed donors for rootstock establishment according to the trait criteria enlisted in the previous section. Their corresponding OP half-sib seedling progenies were grafted with Hass clonal scion to estimate rootstock-transferred h^2 scores for key agronomic traits, such as total number of fruits and fruits' weight after 5 years of production. The estimation of overall h^2 scores (spanning both the mother-offspring and the rootstock-mediated inheritance) for both agronomical traits followed a mothers vs. grafted half-sib offspring linear regression. In this sense, the dependent variable corresponded to the average half-sib family trait values for total number of fruits and fruits' weight measured in avocado cv. Hass trees grafted on top of OP seedling rootstock families obtained from the avocado "criollo" "plus trees." The trait values for the total number of fruits and fruits' weight measured in the original mother "criollo" "plus trees" were depicted in the *X*-axis (**Figure 7**).

The trait total number of fruits presented a slope of 0.18 and an *r* score of 0.16. Since the mothers-offspring heritability corresponded to twice the slope, the h^2 equaled 0.36 (± 0.23). The trait fruits' weight exhibited a slope value of 0.056 with an *r* score of 0.18, and therefore an overall h^2 estimate of 0.11 (± 0.09, **Figure 7**). According to the results obtained, the trait total number of fruits presented a moderate seedling rootstock-mediated heritability of 0.36, indicating the possibility of harnessing natural variation from "criollo" plus trees *via* OP half-sib seedling rootstock families for cv. Hass orchards. On the contrary, the trait fruits' weight presented a lower seedling rootstock-mediated heritability of 0.11, indicating that this trait may be influenced by environmental factors, non-additive genetic factors, such as dominance and epitasis, crop management, and the rootstocks-scion interaction, rather than additive genetic contributions from the OP half-sib rootstock families.

DISCUSSION

We quantified additive genetic parameters across landraces "plus trees" of the super-food fruit tree crop avocado (*P. americana* Mill.), and their OP half-sib seedling families. The SSR markers that were used for the molecular characterization of avocado "criollo" "plus trees" allowed obtaining *G*-BLUP heritability predictions of morphological and agronomic traits. After all, highly polymorphic SSR markers screened across variable genotypes (in terms of their degrees of relationships, from highly related to completely unrelated), enable BLUP predictions, as demonstrated by Reyes-Herrera et al. (2020) in avocado, and Cros et al. (2015, 2019) in oil palm and rubber tree, respectively. This is because pedigree-free marker-based heritability estimations *via* *G*-BLUP work either on the basis of shared relatedness (typically measured as relationships due to recent co-ancestry) or on the basis of genetic hitchhiking due to linkage disequilibrium (LD) among marker loci. In the absence of demonstrated LD (which would require denser single nucleotide polymorphism, SNP markers), shared relatedness as inferred by variable SSR markers across a diverse panel (Ellegren, 2004; Cortés et al., 2011) offer the basis to disclose the phenotypic clustering patterns across family types (which in turn are proportional to heritability scores). In particular, the relationship among samples is critically important, and therefore, was carefully reconstructed by our study *via* the relatedness histogram, the phylogenic relationships among samples, and the unsupervised Bayesian admixed clustering.

We estimated heritability values for 17 morpho-agronomic traits in avocado "criollo" "plus trees" selected as seed donors for rootstocks of the cv. Hass. These values were significant according to their 95% confidence intervals and heritabilities were found to be in a medium range according to Robinson et al. (1951) classification with the magnitude: $0.28 \leq h^2 \geq 0.5$, indicating a moderate possibility of genetic gain through seedling rootstock selection and contribution of these traits to the cv. Hass. Regarding agronomic traits, the heritability of the fruit weight trait was quantified both in avocado "criollo" "plus trees" (0.36) and their OP half-sib seedling progenies used as rootstocks for the Hass scion (0.11), exhibiting a reduction of almost half in heritability. This indicates that nearly 50% of "plus tree" genetic

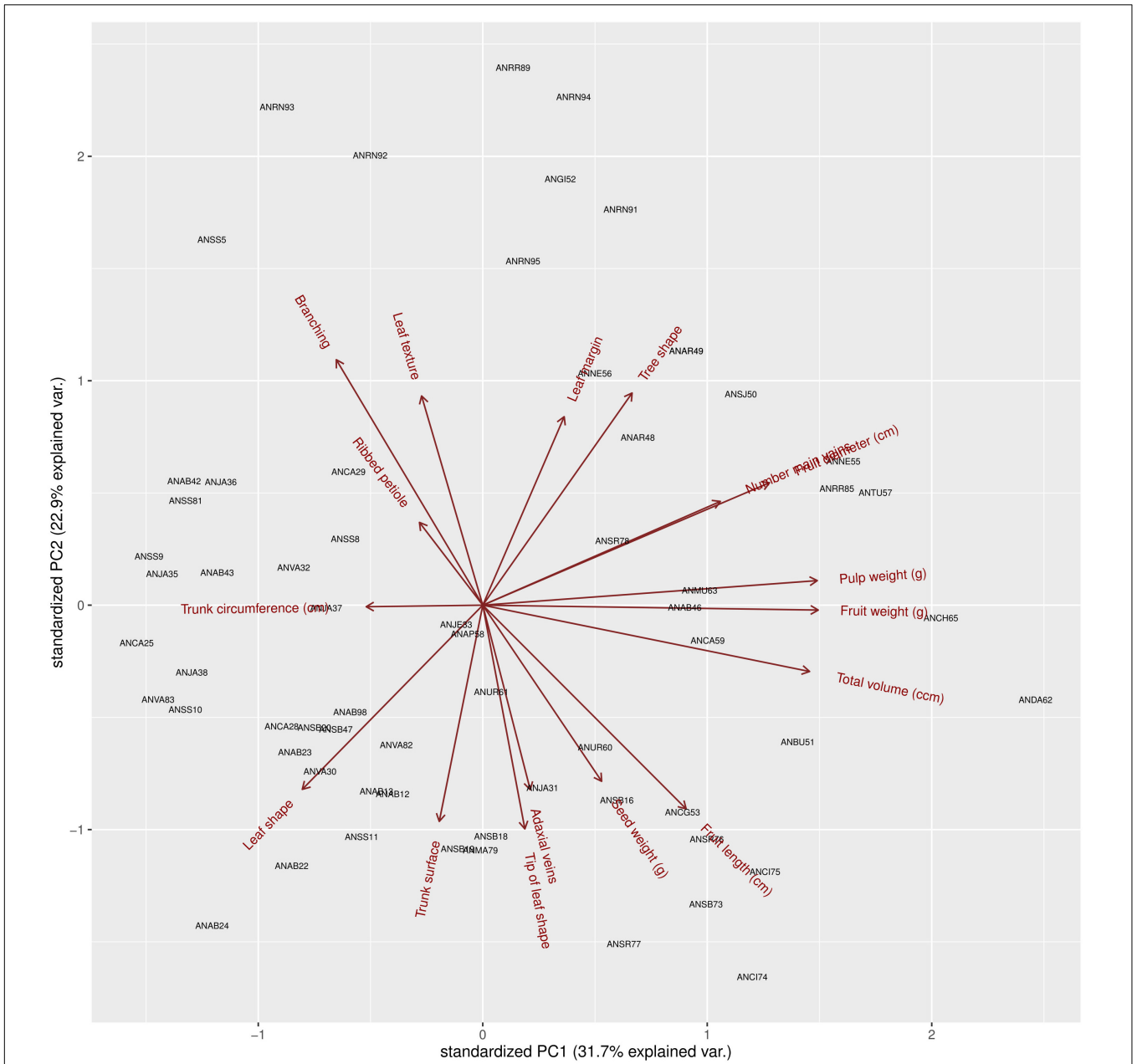

FIGURE 6 | Breeding values for traits (vectors in red) measured in avocado "criollo" "plus trees" (black codes) selected as seed donors for rootstocks of the cv. Hass. A match between the specific tree code and the arrow of the trait vector indicates an additive genetic superiority (a.k.a., breeding value) of that particular tree for the specific trait. Opposing vectors speak for negative genetic correlations, which may limit simultaneous selection for both traits in the same direction, as opposed to orthogonal vectors that suggest traits are free from genetic correlations.

gain for this trait may be inherited to cv. Hass through seed-mediated grafting.

Leveraging Avocado "Criollo" "Plus Trees" as Seed Donors for Rootstock Production

A long-standing debate in the breeding of fruit tree crops is whether seedling rootstocks are capable to enhance productivity in the absence of more uniform clonal rootstocks, which in turn are thought to lack sufficient genetic variability for long-term adaptation. As an intermediate solution, replacing seedlings genotypes by a diverse panel of elite clones is expected to convey major improvements while controlling adaptive genetic erosion (Ingvarsson and Dahlberg, 2019). Nonetheless, avocado cv. Hass plantations in northwest South America have grown exponentially in the last decade without the availability of clonal rootstock genotypes. Instead, they

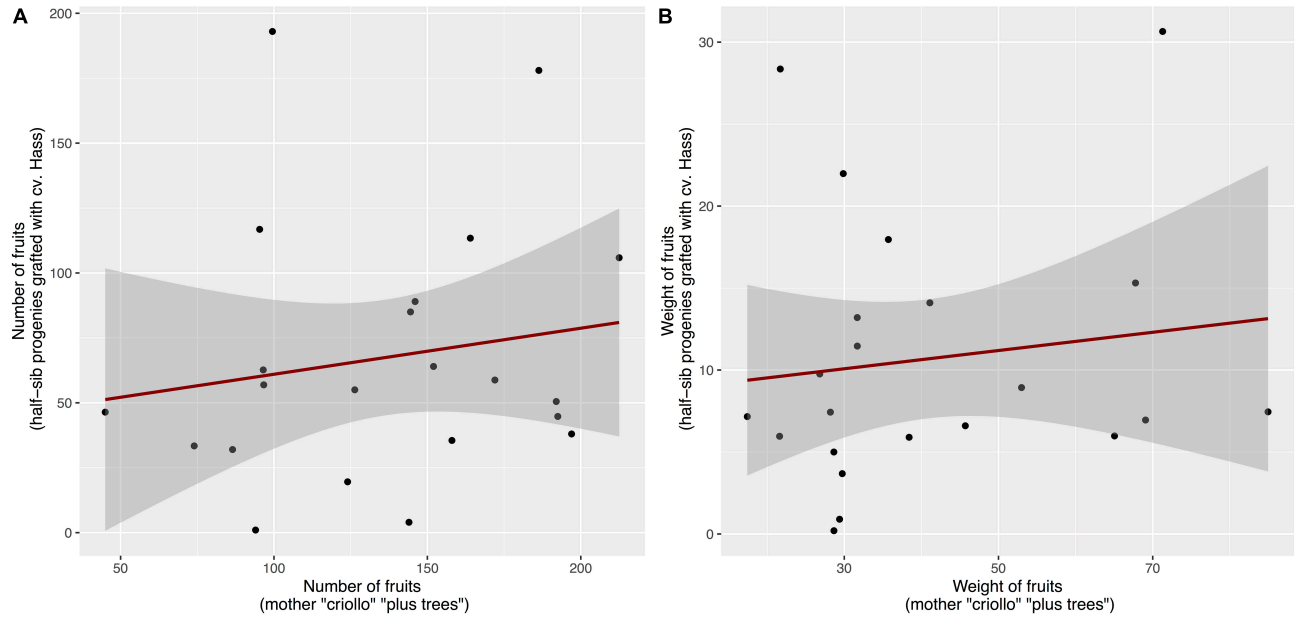

FIGURE 7 | Mother-offspring regressions used to quantify the rootstock-mediated h^2 scores for cumulative **(A)** number of fruits and **(B)** weight of fruits from the Hass clone grafted on OP half-sib seedling progenies of selected avocado "criollo" "plus trees." The h^2 score is computed as twice the slope from the mother-offspring regression (Lynch and Walsh, 1998), and was 0.36 and 0.11 h^2 for number of fruits and weight of fruits, respectively.

have had to rely on cheaper seedling rootstocks from native "criollo" trees without any sort of selection or traceability. Therefore, our narrow sense heritability quantifications are encouraging guides to additive genetic selection of "criollo" "plus trees" as seed donors for rootstock production, conveying up to 35% (h^2_T) of potential genetic gain for a key yield trait as is the total number of fruits. This overall effect totalizes the additive genetic contribution from OP half-sib seedling rootstock families to the Hass clonal scion (i.e., h^2_R = 0.44, as computed by Reyes-Herrera et al., 2020), as well as the expected additive genetic variance putatively inherited from the "criollo" "plus trees" to their OP half-sib F1 seedling offspring (i.e., $h^2_{F1} = h^2_T/h^2_R$ = 0.7). Ultimately, these estimates reinforce the feasibility of utilizing selected avocado "criollo" "plus trees" as seed donors for rootstocks.

Furthermore, the GEBVs, computed here by weighting and standardizing the overall trait superiority by the corresponding heritability scores, are already a robust selection index to target *in situ* superior "criollo" "plus trees" as donors of seedling rootstocks. Alternatively, buds from these selected "criollo" "plus trees" may be grafted to establish *ex situ* seed orchards. In any case, avocado cv. Hass nurseries may still face uncertainty while sorting the OP half-sib offspring from those "criollo" "plus trees" families in the absence of age-age correlations at the seedling stage. To assist early within-family selection of superior half-sib progenies, heritability estimates above 35% (e.g., for total number of fruits) also invite implementing last-generation high-throughput genomic selection (GS) schemes (Desta and Ortiz, 2014; Crossa et al., 2017). Genomic prediction platforms calibrate infinitesimal polygenic additive models (Cortés et al., 2020) capable to forecast breeding values for low-heritability complex traits (Klápště et al., 2020) at early life stages (Grattapaglia et al., 2018). Therefore, GS simultaneously makes indirect selection more precise, while allowing speeding up breeding cycles. Its potential in perennials with prolonged juvenile phases has already been industrially scaled up by the forest tree sector (Resende et al., 2012, 2020; Tan et al., 2017; Cappa et al., 2019; Thistlethwaite et al., 2019), with some prospective pilot studies carried out in temperate fruit trees (Kumar et al., 2012, 2019, 2020; Muranty et al., 2015; Iwata et al., 2016; Ferrão et al., 2021). Based on this, avocado cv. Hass nurseries might exploit the potential of GS to choose the superior progenies from the "criollo" "plus trees," even before grafting. Such innovation will optimize timing and monetary resources at nurseries, while ultimately translating into reduced tree mortality and increased productivity/adaptability during the establishment and production phases.

Meanwhile, efforts must not be spared to breed a diverse panel of elite clonal rootstock genotypes resistant to root rot (*P. cinnamomi*), a major fungal threat for avocado plantation worldwide (Toapanta-Gallegos et al., 2017; Sánchez-González et al., 2019), while keeping high productivity and adaptation across heterogeneous mountain climates (Cortés and Wheeler, 2018). However, breeding elite clonal tree genotypes with conventional direct phenotypic selection doubles the breeding cycle length compared to gradual population recurrent selection due to longer progeny-testing and more clonal trials (Neale and Kremer, 2011). Therefore, GS may once more assist the identification of elite clonal rootstocks for the avocado cv.

Hass industry, as already exemplified by the forestry sector by reducing the length of the clonal breeding cycle to 50% (Resende et al., 2012). Parallel efforts must be conveyed to better standardize the propagation of clonal rootstocks [e.g., *via* genotype-dependent micro-cloning (Ernst, 1999) and double grafting (Frolich and Platt, 1971) protocols], which is still a major bottleneck in regions with high availability of native avocado trees capable to source nurseries with cheaper seedlings.

Paving the Path Toward Predictive Rootstock Breeding

The h^2 and GEBVs estimate gathered as part of this study encourage the implementation of more systematic genetic-guided indirect selection platforms both as part of early (before grafting) seedling screening at nurseries of avocado cv. Hass, as well as within the avocado rootstock breeding cycles *per se*. Nonetheless, a question persists, which is whether the studied SSR molecular markers are enough to capture sufficient additive variance as to make predictive breeding reliable. This potentially remains open judging by the r scores (> 0.54) of our pedigree-free SSR-inferred estimation of genetic parameters (Frentiu et al., 2008; Wilson et al., 2010), which embraced sufficient variation across distinct kinship levels (Milner et al., 2000; Kruuk, 2004; Berenos et al., 2014). Therefore, avocado rootstock breeders would be able to leverage SSR characterizations across a diverse panel of avocado accessions (Alcaraz and Hormaza, 2007; Cañas-Gutiérrez et al., 2015; Ferrer-Pereira et al., 2017; Boza et al., 2018; Cañas-Gutiérrez et al., 2019b; Reyes-Herrera et al., 2020; Sánchez-González et al., 2020). SSR-inferred relationships also allow bridging the difficulty to generate complete pedigrees in tropical avocados. It is challenging to carry out control crossing in tropical "criollo" avocados because their pollination is open, and exhibit dichogamous protogyny. It would also be unviable to gather complete pedigrees from *in situ* "criollo" avocado trees because they exhibit disparity in the phenological phases, and are situated in remote locations where access is limited to perform controlled monitoring. This is why our team is currently bringing buds from the "criollo" trees for grafting on their own half-sib rootstocks. This strategy would allow cloning them at a single research station to better study their phenology and control their pollination. Meanwhile, molecular markers offer a realistic pathway to reconstruct relatedness among avocado "criollo" trees. The prospect to utilize highly polymorphic SSRs for predictive breeding across a diverse panel of genotypes spanning various levels of relatedness is also in line with previous proof-of-concept pilot studies carried out in oil palm (Cros et al., 2015) and rubber tree (Cros et al., 2019). This is because indirect selection works either on the basis of shared relatedness (typically measured as kinship relationships due to recent co-ancestry, Sedlacek et al., 2016) or on the basis of linkage disequilibrium (LD) between marker loci and the genetic variants that underlie phenotypic variation (Thistlethwaite et al., 2020). The high mutation rate of SSRs (Ellegren, 2004), and consequently, its unsurpassed polymorphism information content (Cortés et al., 2011; Blair et al., 2012), efficiently disclose the shared co-ancestry among genotypes (Lynch and Ritland, 1999) and its ultimate potential for predictive breeding.

Nonetheless, predictive breeding would be incapable to rely on LD (Blair et al., 2018) between markers and the genetic variants that underlie trait variation because SSRs are insufficient to reconstruct the polygenic architecture of complex (Hirschhorn and Daly, 2005) rootstock-mediated traits. After all, SSR markers, despite polymorphic, are rare across the genome, which makes them unlikely to be found in LD with any causal variant (Slatkin, 2008). To bridge this gap, future research will have to couple SNP screening of avocado genotypes (Kuhn et al., 2019; Rendón-Anaya et al., 2019; Rubinstein et al., 2019; Talavera et al., 2019), *via* genotyping-by-sequencing (GBS, Elshire et al., 2011; Cortés and Blair, 2018), whole-genome re-sequencing (WGR, Fuentes-Pardo and Ruzzante, 2017) and RNAseq (Jensen et al., 2012; Sun, 2012; Reeksting et al., 2016), with genome-wide association studies (GWAS, Khan and Korban, 2012). These innovative "omic" approaches should also target diverse avocado "criollo" "plus trees," their OP half-sib progenies used as rootstocks for the Hass scion, and different tissues of the grafted tree, for instance through single-cell sequencing (Tang et al., 2019). Combined genetic mapping efforts will in turn inform on the upstream regulatory gene networks that contribute shaping the rootstock—scion compatibility (Loupit and Cookson, 2020) and interaction (Albacete et al., 2015; Warschefsky et al., 2016; Migicovsky and Myles, 2017), as well as on the corresponding downstream mechanistic physiological processes [e.g., transport of water and nutrients from the rootstocks, and large-scale movement of hormones, proteins, mRNAs, and sRNAs between the rootstock and the scion (Wang et al., 2017; Cookson et al., 2019)].

PERSPECTIVES

The current research has proven the feasibility to transfer genetic superiority from native avocado "criollo" "plus trees" to commercial Hass plantations *via* OP half-sib seedling rootstock families. Specifically, our estimates suggest that this seedling rootstock breeding strategy may convey up to 35% of genetic gain for a relevant yield trait, as is the total number of fruits. Still, genetic-guided selection of "criollo" "plus trees" as donors of seedling rootstocks is also likely to impact other relevant traits both at the rootstock and scion levels. For instance, alterable rootstock phenotypes could include biotic resistance (Clavijo and Holguiín, 2020; Ramírez-Gil et al., 2020a, Guevara-Escudero et al., 2021) to root rot *P. cinnamomi* (Smith et al., 2011; Reeksting et al., 2016; Sánchez-González et al., 2019), drought and heat tolerance (López-Hernández and Cortés, 2019), mineral nutrient uptake (Bard and Wolstenholme, 1997; Calderón-Vázquez et al., 2013; Tamayo-Vélez and Osorio, 2018; Tamayo-Vélez et al., 2018), tolerance to salinity (Bernstein et al., 2001; Mickelbart and Arpaia, 2002; Raga et al., 2014), genetic compatibility (López-Guzmán et al., 2021), and harvest (Ramírez-Gil et al., 2020b) and postharvest physiochemical parameters (Astudillo and Rodríguez, 2018). This extended panel of potential rootstock

effects is key for the avocado industry because cv. Hass lacks trait variation for resistance to diseases (e.g., it is susceptibility to *P. cinnamomi*, Sánchez-González et al., 2019). Therefore avocado cv. Hass is unable to provide a realistic source of rootstocks for commercial plantations, especially in soil types with a high natural incidence of *P. cinnamomi*. However, from a theoretical purely academic point of view, Hass grafted on Hass would be an appealing reference control treatment for future rootstock × scion factorial designs.

Meanwhile, indirect rootstock effects on scion's traits may also involve alternate bearing and nutrition (Mickelbart et al., 2007), carbohydrate accumulation (Whiley and Wolstenholme, 1990), postharvest anthracnose development (Willingham et al., 2001), overall trunk high, and fruit production for the exportation market with acceptable fruit weight and lack of thrips' damage (Reyes-Herrera et al., 2020). Therefore, future research efforts should aim quantifying, across this expanded repertoire of adaptive, morphological, harvest, and quality traits, combined h^2 and GEBVs estimates that jointly account for the additive inheritance of phenotypic variance from the "plus trees" seedling donors to their OP progenies (i.e., family selection), as well as from these half-sib rootstock families to the avocado cv. Hass clonal scion (i.e., within-family offspring selection). These future studies should also envision collecting further data across years (beyond the 4 years production data for *in situ* "criollo" "plus trees", and the 5 years production of the OP half-sib seedling rootstock progenies grafted with the cv. Hass clone). Altogether, these efforts will permit strengthening our modeling effort. Such baseline knowledge will further allow implementing predictive breeding platforms to guide before-grafting seedling selection at nurseries, while targeting diverse climates (Costa-Neto et al., 2020) as part of an "enviromic prediction" (Resende et al., 2020).

Diversifying selection of half-sib rootstock families and within-family offspring at seedling nurseries remains a promising and affordable avenue, especially if coupled with indirect genetic-guided predictors. Seedling rootstock breeding also enables broadening the genetic basis of young avocado plantations in regions with diverse sources of native avocado trees, some of them still cultivated in backyard gardens, traditional orchards, and as living fences (Galindo-Tovar et al., 2007). Yet, selection of diverse clonal rootstock genotypes may be a more appealing long-term strategy to contribute diminishing tree mortality by conveying genotypic superiority and phenotypic uniformity, while controlling genetic erosion (Ingvarsson and Dahlberg, 2019). Fulfilling the increased demand of diverse elite clonal rootstocks is becoming a pivotal requirement at avocado nurseries (Quintero et al., 2021), which could benefit by merging current rootstock breeding programs with predictive breeding platforms capable to boost selection accuracy of clonal rootstocks in shorter cycles (Resende et al., 2012).

CONCLUSION

Our work has innovated the implementation of a pedigree-free marker-inferred heritability prediction model to assess the potential of avocado "criollo" "plus trees" as seed donors to produce OP half-sib rootstock families for the Hass clonal scion. The h^2 scores and GEBVs gathered throughout this research indicate that the selection of seed donor "criollo" trees is able to confer, *via* seedling rootstocks, up to 35% of genetic gain for relevant yield traits, such as total number of fruits. This finding is in line with the overwhelming indirect rootstock effects on the avocado cv. Hass scion phenotype, which has been reported to impact a wide spectrum of harvest and quality traits beyond the total number of fruits (up to 44%), such as number of fruits with exportation quality, as well as those discarded because of low weight or thrips" damage. The feasibility to harness "criollo" seedling diversity within a rootstock-breeding program reinforces the utility of native avocado trees to source cryptic genetic variation and adaptive potential (Cortés and López-Hernández, 2021), ultimately counterbalancing the winnowing effect (Cortés et al., 2022) of clonal rootstocks and scions on genetic variation in fruit tree plantations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

AUTHOR CONTRIBUTIONS

GC-G, AN-A, and AC conceived the original sampling and research questions. GC-G and SS-O performed phenotypic data collection and sampling of leaves for genotyping. GC-G carried out DNA extractions, SSR genotyping, and alleles size estimation. GC-G, FL-H, and AC filtered input datasets and carried out data analyses. FL-H and AC plotted diagrams. GC-G, FL-H, AN-A, and AC interpreted heritability scores. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by grants from Sistema General de Regalías (SGR—Antioquia) awarded to AN-A under contract numbers 1833 and 80740-096-2021. MinCiencias' postdoctoral fellowship 891 was also acknowledged for subsidizing GC-G's internship in AGROSAVIA during 2021 under the supervision of AC and AN-A. Permiso Marco 1466-2014 of AGROSAVIA allowed the sampling of the avocado "criollo" "plus trees" in the province of Antioquia, Colombia. AGROSAVIA's Department for Research Capacity Building is recognized for sponsoring the participation of the last author in the V Latin American Avocado Congress (held on September 2017 in Ciudad Guzmán, Mexico), and the 3rd Global Congress on Plant Biology and Biotechnology (held on March 2019 in Singapore), where some preliminary hypotheses sustaining this work were discussed. AGROSAVIA's Research Department, through its Scientific Divulgation Unit, and the editorial office of Frontiers in Plant Science are deeply acknowledged for subsidizing the article processing charge (APC).

ACKNOWLEDGMENTS

We appreciate the help of all the people who assisted during the sampling of avocado "criollo" trees. Specifically, field assistants M. Osorno, G. Higinio, L. Cano, and M. Latorre are also deeply thanked for monitoring and data collection in the avocado elite collection at AGROSAVIA's La Selva research station. We would like to acknowledge M. Londoño, too, for the invitation to socialize some of the findings presented in this work at Territorio Aguacate (held on December 2021 in Medellín, Colombia), and the IX World Avocado Congress (held on September 2019 in Medellín, Colombia). Furthermore, we highly appreciate discussions with A. Barrientos, J.I. Hormaza, M.F. Martínez, P. Manosalva, R. Martínez, D. Monsalve, and P. Tamayo on avocado rootstock breeding, as well as with J. Berdugo-Cely, I. Cerón-Souza, P.H. Reyes-Herrera, and R. Yockteng on avocado "criollo" diversification and genomics. M. Torres is thanked for assistamce. We are in debt with AGROSAVIA's Research Department (and its Scientific Divulgation Unit) for supporting this article's processing charge (APC). Last but not least, 2021 mourns the passing of two avocado visionaries: Samuel Salazar-García (INIFAP. Santiago Ixcuintla, Nayarit, Mexico), and André Ernst (Allesbeste Boerdery. Tzaneen, Limpop, South Africa). Among many outstanding contributions, we would like to highlight their efforts to select dwarfing avocado rootstocks tolerant to drought and *P. cinnamomi* (Salazar-García et al., 2004; Herrera-González et al., 2013), and implement clonal propagation of avocado rootstocks (Ernst, 1999), works that ultimately contributed to the foundation of modern avocado rootstock breeding.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.843099/full#supplementary-material

Supplementary Figure 1 | Distance tree computed by means of Neighbor Joining algorithm in R with 10,000 bootstrap replicates.

Supplementary Table 1 | Avocado "criollo" "plus trees" considered as part of this study. ID number, municipality, sub-region within the province of Antioquia, and elevation are depicted in each case. A preliminary version of this table was originally compiled by Cañas-Gutiérrez et al. (2019b). Under the last column, the symbol + marks trees for which complete molecular and phenotyping characterizations were possible. Under the same column, the asterisk * marks "criollo" "elite trees" that donated seeds for the establishment of OP half-sib rootstock families grafted with the cv. Hass clone as part of the common garden (provenance trial) established at CI La Selva (Rionegro, Antioquia, 2,100 m asl).

Supplementary Table 2 | Codification of qualitative traits (**Table 1**) measured in the avocado "criollo" selected "plus trees," based on descriptors of the International Plant Genetic Resources Institute (IPGRI).

Supplementary Table 3 | Primers and motif pattern for the 13 microsatellite markers (simple sequence repeats—SSRs) used in this study to avocado "criollo" "plus tree." Forward and reverse primers, sequence motif, source, and summary statistics are shown. Markers were originally designed by Sharon et al. (1997) and Ashworth et al. (2004) and were prioritized according to their polymorphism information content (PIC), following Alcaraz and Hormaza (2007).

REFERENCES

Ahsan, M. U., Hayward, A., Alam, M., Bandaralage, J. H., Topp, B., Beveridge, C. A., et al. (2019). Scion control of miRNA abundance and tree maturity in grafted avocado. *BMC Plant Biol.* 19:382. doi: 10.1186/s12870-019-1994-5

Albacete, A., Martinez-Andujar, C., Martinez-Perez, A., Thompson, A. J., Dodd, I. C., and Perez-Alfocea, F. (2015). Unravelling rootstock X scion interactions to improve food security. *J. Exp. Bot.* 66, 2211–2226. doi: 10.1093/jxb/erv027

Alcaraz, M. L., and Hormaza, J. I. (2007). Molecular characterization and genetic diversity in an avocado collection of cultivars and local Spanish genotypes using SSRs. *Hereditas* 144:253. doi: 10.1111/j.2007.0018-0661.02019x

Amiri, M. E., Fallahi, E., and Safi-Songhorabad, M. (2014). Influence of rootstock on mineral uptake and scion growth of 'Golden Delicious'and 'Royal Gala'apples. *J. Plant Nutr.* 37, 16–29. doi: 10.1080/01904167.2013.792838

Arenas, S., Cortés, A. J., Mastretta, A., and Jaramillo, J. P. (2021). Evaluating the accuracy of genomic prediction for the management and conservation of relictual natural tree populations. *Tree Genet. Genomes* 17:12. doi: 10.1007/s11295-020-01489-1

Ashworth, V. E. T. M., Kobayashi, M. C., De la Cruz, M., and Clegg, M. T. (2004). Microsatellite markers in avocado (*Persea americana* Mill.): development of dinucleotide and trinucleotide markers. *Sci. Hortic.* 101, 255–267. doi: 10.1016/j.scienta.2003.11.008

Astudillo, C., and Rodríguez, P. (2018). Physicochemical parameters of avocado *Persea americana* mill. Cv. hass (Lauraceae) grown in Antioquia (Colombia) for export. *Cienc. Tecnol. Agropecu.* 19, 383–392. doi: 10.21930/rcta.vol19_num2_art:694

Balducci, F., Capriotti, L., Mazzoni, L., Medori, I., Albanesi, A., Giovanni, B., et al. (2019). The rootstock effects on vigor, production and fruit quality in sweet cherry (*Prunus Avium* L.). *J. Berry Res.* 9, 249–265. doi: 10.3233/JBR-180345

Bard, Z. J., and Wolstenholme, B. N. (1997). Soil boron application for the control of boron deficiency in Kwazulu-Natal avocado orchards. *South African Avocado Growers'. Assoc. Yearbook* 20, 13–15.

Berenos, C., Ellis, P. A., Pilkington, J. G., and Pemberton, J. M. (2014). Estimating quantitative genetic parameters in wild populations: a comparison of pedigree and genomic approaches. *Mol. Ecol.* 23, 3434–3451. doi: 10.1111/mec.12827

Bernal, E., Alonso, J., and Díaz, D. C. (2014). *Manejo del Cultivo. En: Corporación Colombiana de Investigación Agropecuaria – Corpoica; Secretaria de Agricultura y Desarrollo Rural de Antioquia. Actualización Tecnológica y Buenas Prácticas Agrícolas (BPA) en el Cultivo de Aguacate*, 2nd Edn. Medellín: Agrosiaa, 10–151.

Bernstein, N., Loffe, M., and Zilberstaine, M. (2001). Salt-stress effects on avocado rootstock growth. I. Establishing criteria for determination of shoot growth sensitivity to the stress. *Plant Soil* 233, 1–11. doi: 10.1023/A:1010370802773

Blair, M. W., Cortes, A. J., Farmer, A. D., Huang, W., Ambachew, D., Penmetsa, R. V., et al. (2018). Uneven recombination rate and linkage disequilibrium across a reference SNP map for common bean (*Phaseolus vulgaris* L.). *PLoS One* 13:e0189597. doi: 10.1371/journal.pone.0189597

Blair, M. W., Soler, A., and Cortés, A. J. (2012). Diversification and population structure in common beans (*Phaseolus vulgaris* L.). *PLoS One* 7:e49488. doi: 10.1371/journal.pone.0049488

Boza, E., Tondo, C., Ledesma, N., Campbell, R., Bost, J., Schnell, R., et al. (2018). Genetic differentiation, races and interracial admixture in avocado (*Persea americana* Mill.), and *Persea* Spp. evaluated using SSR markers. *Genetic Resour. Crop Evolut.* 65, 1195–1215. doi: 10.1007/s10722-018-0608-7

Calderón-Vázquez, C., Durbin, M. L., Ashworth, V. E. T. M., Livia, T., Meyer, K. K. T., and Clegg, M. T. (2013). Quantitative genetic analysis of three important nutritive traits in the fruit of avocado. *J. Am. Soc. Hortic. Sci.* 138, 283–289. doi: 10.21273/JASHS.138.4.283

Cañas-Gutiérrez, G. P., Alcaraz, L., Hormaza, J. I., Arango-Isaza, R. E., and Saldamando-Benjumea, C. I. (2019a). Diversity of avocado (*Persea americana* Mill.) cultivars from Antioquia (Northeast Colombia) and comparison with a worldwide germplasm collection. *Turk. J. Agric. For.* 43, 437–449. doi: 10.3906/tar-1807-25

Cañas-Gutiérrez, G. P., Arango-Isaza, R. E., and Saldamando-Benjumea, C. I. (2019b). Microsatellites revealed genetic diversity and population structure

in Colombian avocado (*Persea americana* Mill.) germplasm collection and its natural populations. *J. Plant Breed. Crop Sci.* 11, 106–119. doi: 10.5897/JPBCS2018.0792

Cañas-Gutiérrez, G. P., Galindo-López, L. F., Arango-Isaza, R., and Saldamando-Benjumea, C. I. (2015). Diversidad genética de cultivares de aguacate (*Persea americana* Mill.) en Antioquia, Colombia. *Agron. Mesoam.* 26:129. doi: 10.15517/am.v26i1.16936

Cappa, E. P., De Lima, B. M., Da Silva-Junior, O. B., Garcia, C. C., Mansfield, S. D., and Grattapaglia, D. (2019). Improving genomic prediction of growth and wood traits in *eucalyptus* using phenotypes from non-genotyped trees by single-step Gblup. *Plant Sci.* 284, 9–15. doi: 10.1016/j.plantsci.2019.03.017

Chen, Z. Q., Baison, J., Pan, J., Karlsson, B., Andersson, B., Westin, J., et al. (2018). Accuracy of genomic selection for growth and wood quality traits in two control-pollinated progeny trials using exome capture as the genotyping platform in Norway spruce. *BMC Genomics* 19:946. doi: 10.1186/s12864-018-5256-y

Clavijo, A. P., and Holguiín, C. M. (2020). Pathogenicity of commercial entomopathogenic fungal strains on the avocado seed borer (Asb), *Heilipus lauri* (Coleoptera: Curculionidae) under laboratory conditions. *Int. J. Trop. Insect Sci.* 40, 1059–1067. doi: 10.1007/s42690-020-00167-y

Cookson, S. J., Prodhomme, D., Chambaud, C., Hévin, C., Valls Fonayet, J., Hilbert, G., et al. (2019). Understanding scion-rootstock interactions at the graft interface of grapevine. *Acta Hortic.* 1248, 369–374. doi: 10.17660/ActaHortic.2019.1248.53

Cortés, A. J., and Blair, M. W. (2018). Genotyping by sequencing and genome – environment associations in wild common bean predict widespread divergent adaptation to drought. *Front. Plant Sci.* 9:128. doi: 10.3389/fpls.2018.00128

Cortés, A. J., Chavarro, M. C., and Blair, M. W. (2011). Snp marker diversity in common bean (*Phaseolus vulgaris* L.). *Theor. Appl. Genet.* 123, 827–845. doi: 10.1007/s00122-011-1630-8

Cortés, A. J., Cornille, A., and Yockteng, R. (2022). Evolutionary genetics of crop-wild complexes. *Genes* 13:1. doi: 10.3390/genes13010001

Cortés, A. J., and López-Hernández, F. (2021). Harnessing crop wild diversity for climate change adaptation. *Genes* 12:783. doi: 10.3390/genes12050783

Cortés, A. J., Restrepo-Montoya, M., and Bedoya-Canas, L. E. (2020). Modern strategies to assess and breed forest tree adaptation to changing climate. *Front. Plant Sci.* 11:583323. doi: 10.3389/fpls.2020.583323

Cortés, A. J., and Wheeler, J. A. (2018). "The environmental heterogeneity of mountains at a fine scale in a changing world," in *Mountains, Climate, and Biodiversity*, eds C. Hoorn, A. Perrigo, and A. Antonelli (New York, NY: Wiley).

Costa-Neto, G., Fritsche-Neto, R., and Crossa, J. (2020). Nonlinear kernels, dominance, and envirotyping data increase the accuracy of genome-based prediction in multi-environment trials. *Heredity* 126, 92–106. doi: 10.1038/s41437-020-00353-1

Cros, D., Denis, M., Sánchez, L., Cochard, B., Flori, A., Durand-Gasselin, T., et al. (2015). genomic selection prediction accuracy in a perennial crop: case study of oil palm (*Elaeis guineensis* Jacq.). *Theor. Appl. Genet.* 128, 397–410. doi: 10.1007/s00122-014-2439-z

Cros, D., Mbo-Nkoulou, L., Bell, J. M., Oum, J., Masson, A., Soumahoro, M., et al. (2019). Within-family genomic selection in rubber tree (*Hevea brasiliensis*) increases genetic gain for rubber production. *Ind. Crops Prod.* 138:111464. doi: 10.1016/j.indcrop.2019.111464

Crossa, J., Perez-Rodriguez, P., Cuevas, J., Montesinos-Lopez, O., Jarquin, D., De Los Campos, G., et al. (2017). Genomic selection in plant breeding: methods: models, and perspectives. *Trends Plant Sci.* 22, 961–975. doi: 10.1016/j.tplants.2017.08.011

de los Campos, G., Naya, H., Gianola, D., Crossa, J., Legarra, A., Manfredi, E., et al. (2009). Predicting quantitative traits with regression models for dense molecular markers and pedigree. *Genetics* 182, 375–385. doi: 10.1534/genetics.109.101501

de los Campos, G., Sorensen, D., and Gianola, D. (2015). Genomic heritability: what is it? *PLoS Genet.* 11:e1005048. doi: 10.1371/journal.pgen.1005048

Desta, Z. A., and Ortiz, R. (2014). Genomic selection: genome-wide prediction in plant improvement. *Trends Plant Sci.* 19, 592–601. doi: 10.1016/j.tplants.2014.05.006

Dieters, M. J., White, T. L., and Hodge, G. R. (1995). Genetic parameter estimates for volume from full-sib tests of slash pine (*Pinus elliottii*). *Can. J. Forest Res.* 25, 1397–1408. doi: 10.1139/x95-152

Egea, J., Ruiz, D., and Martínez-Gómez, P. (2004). Influence of rootstock on the productive behaviour of 'Orange Red' apricot under Mediterranean conditions. *Fruits* 59, 367–373. doi: 10.1051/fruits:2004035

Ellegren, H. (2004). Microsatellites: simple sequences with complex evolution. *Nat. Rev. Genet.* 5, 435–445. doi: 10.1038/nrg1348

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., et al. (2011). A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One* 6:e19379. doi: 10.1371/journal.pone.0019379

Ernst, A. A. (1999). Micro cloning: a multiple cloning technique for avocados using micro containers. *Rev. Chapingo Ser. Hortic.* 5, 217–220.

Expósito, A., Pujolà, M., Achaerandio, I., Giné, A., Escudero, N., Fullana, A. M., et al. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. *Front. Plant Sci.* 11:560024. doi: 10.3389/fpls.2020.560024

Falconer, D. S., Mackay, T. F., and Frankham, R. (1996). Introduction to quantitative genetics (4th edn). *Trends Genet.* 12:280. doi: 10.1016/0168-9525(96)81458-2

Fernandez-Paz, J., Cortés, A. J., Hernández-Varela, C. A., Mejía-de-Tafur, M. S., Rodríguez-Medina, C., and Baligar, V. C. (2021). Rootstock mediated genetic variance in cadmium uptake by juvenile cacao (*Theobroma cacao* L.) genotypes, and its effect on growth and physiology. *Front. Plant Sci.* 12:777842. doi: 10.3389/fpls.2021.777842

Ferrão, L. F. V., Amadeu, R. R., Benevenuto, J., De Bem Oliveira, I., and Munoz, P. R. (2021). Genomic selection in an outcrossing autotetraploid fruit crop: lessons from blueberry breeding. *Front. Plant Sci.* 12:676326. doi: 10.3389/fpls.2021.676326

Ferrer-Pereira, H., Pérez-Almeida, I., Raymúndez-Urrutia, M., and Suárez, L. (2017). Genetic relationship analysis for avocado cultivars from Venezuelan germplasm bank (INIA-CENIAP) using molecular markers. *Tree Genet. Genomes* 13:82. doi: 10.1007/s11295-017-1164-6

Frentiu, F. D., Clegg, S. M., Chittock, J., Burke, T., Blows, M. W., and Owens, I. P. F. (2008). Pedigree-free animal models: the relatedness matrix reloaded. *Proc. R. Soc. B Biol. Sci.* 275, 639–647. doi: 10.1098/rspb.2007.1032

Frolich, E. F., and Platt, R. G. (1971). Use of the etiolation technique in rooting avocado cuttings. *Calif. Avocado Soc.* 55, 97–109.

Fuentes-Pardo, A. P., and Ruzzante, D. E. (2017). Whole-genome sequencing approaches for conservation biology: advantages, limitations and practical recommendations. *Mol. Ecol.* 26, 5369–5406. doi: 10.1111/mec.14264

Galindo-Tovar, M. E., Arzate-Fernández, A. M., Ogata-Aguilar, N., and Landero-Torres, I. (2007). The avocado (*Persea americana*, Lauraceae) crop in Mesoamerica: 10,000 years of history. *Harv. Pap. Bot.* 12, 325–334. doi: 10.3100/1043-4534(2007)12[325:TAPALC]2.0.CO;2

Giorgi, M., Capocasa, F., Scalzo, J., Murri, G., Battino, M., and Mezzetti, B. (2005). The rootstock effects on plant adaptability, production, fruit quality, and nutrition in the peach (Cv. 'Suncrest'). *Sci. Horticul.* 107, 36–42. doi: 10.1016/j.scienta.2005.06.003

Goldschmidt, E. E. (2014). Plant grafting: new mechanisms, evolutionary implications. *Front. Plant Sci.* 5:727. doi: 10.3389/fpls.2014.00727

Grattapaglia, D., Silva-Junior, O. B., Resende, R. T., Cappa, E. P., Muller, B. S. F., Tan, B., et al. (2018). Quantitative genetics and genomics converge to accelerate forest tree breeding. *Front. Plant Sci.* 9:1693. doi: 10.3389/fpls.2018.01693

Guevara-Escudero, M., Osorio, A. N., and Cortés, A. J. (2021). Integrative pre-breeding for biotic resistance in forest trees. *Plants* 10:2022. doi: 10.3390/plants10102022

Gullo, G., Motisi, A., Zappia, R., Dattola, A., Diamanti, J., and Mezzetti, B. (2014). Rootstock and fruit canopy position affect peach [*Prunus persica* (L.) Batsch] (Cv. Rich May) plant productivity and fruit sensorial and nutritional quality. *Food Chem.* 153, 234–242. doi: 10.1016/j.foodchem.2013.12.056

Gutierrez, A. P., Matika, O., Bean, T. P., and Houston, R. D. (2018). Genomic selec- tion for growth traits in Pacific oyster (*Crassostrea gigas*): potential of low-density marker panels for breeding value prediction. *Front. Genet.* 9:391. doi: 10.3389/fgene.2018.00391

Herrera-González, J. A., Salazar-García, S., Gutiérrez-Martínez, P., and González-Durán, I. J. L. (2013). Postharvest performance of 'Hass' avocado fruit is influenced by rootstock. *Rev. Mex. Cienc. Agríc.* 4, 19–32.

Hirschhorn, J. N., and Daly, M. J. (2005). Genome-wide association studies for common diseases and complex traits. *Nat. Rev. Genet.* 6, 95–108. doi: 10.1038/nrg1521

Holland, J. B., Nyquist, W. E., and Cervantes-MartiìNez, C. T. (2003). Estimating and interpreting heritability for plant breeding: an update. *Plant Breed. Rev.* 22, 9–112. doi: 10.1002/9780470650202.ch2

Hormaza, J. I. (2020). *Una Visión Sobre las Variedades y Portainjertos en Aguacates*. Available online at: redagricola.com/co/una-vision-sobre-las-variedades-y-portainjertos-en-aguacates/ (accessed May 22, 2020).

Ingvarsson, P. K., and Dahlberg, H. (2019). The effects of clonal forestry on genetic diversity in wild and domesticated stands of forest trees. *Scand. J. For. Res.* 34, 370–379. doi: 10.1080/02827581.2018.1469665

Iwata, H., Minamikawa, M. F., Kajiya-Kanegae, H., Ishimori, M., and Hayashi, T. (2016). Genomics-assisted breeding in fruit trees. *Breed. Sci.* 66, 100–115. doi: 10.1270/jsbbs.66.100

Jensen, P. J., Halbrendt, J., Fazio, G., Makalowska, I., Altman, N., Praul, V., et al. (2012). Rootstock-regulated gene expression patterns associated with fire blight resistance in apple. *BMC Genomics* 13:9. doi: 10.1186/1471-2164-13-9

Kamvar, Z. N., Tabima, J. F., and Grünwald, N. J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2:e281. doi: 10.7717/peerj.281

Khan, M. A., and Korban, S. S. (2012). Association mapping in forest trees and fruit crops. *J. Exp. Bot.* 63, 4045–4060.

Klápště, J., Dungey, H. S., Telfer, E. J., Suontama, M., Graham, N. J., Li, Y., et al. (2020). Marker selection in multivariate genomic prediction improves accuracy of low heritability traits. *Front. Genet.* 11:499094. doi: 10.3389/fgene.2020.499094

Kruuk, L. E. B. (2004). Estimating genetic parameters in natural populations using the "animal model". *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 359, 873–890. doi: 10.1098/rstb.2003.1437

Kuhn, D. N., Livingston, D. S., Richards, J. H., Manosalva, P., Van Den Berg, N., and Chambers, A. H. (2019). Application of genomic tools to avocado (*Persea americana*) breeding: SNP discovery for genotyping and germplasm characterization. *Sci. Hortic.* 246, 1–11. doi: 10.1016/j.scienta.2018.10.011

Kumar, S., Chagne, D., Bink, M. C., Volz, R. K., Whitworth, C., and Carlisle, C. (2012). Genomic selection for fruit quality traits in apple (*Malus x domestica* Borkh.). *PLoS One* 7:e36674. doi: 10.1371/journal.pone.0036674

Kumar, S., Hilario, E., Deng, C. H., and Molloy, C. (2020). Turbocharging introgression breeding of perennial fruit crops: a case study on apple. *Hortic. Res.* 7:47. doi: 10.1038/s41438-020-0270-z

Kumar, S., Kirk, C., Deng, C. H., Shirtliff, A., Wiedow, C., Qin, M., et al. (2019). Marker-trait associations and genomic predictions of interspecific pear (*Pyrus*) fruit characteristics. *Sci. Rep.* 9:9072. doi: 10.1038/s41598-019-45618-w

Kviklys, D., and Samuoliene, G. (2020). Relationships among the rootstock, crop load, and sugar hormone signaling of apple tree, and their effects on biennial bearing. *Front. Plant Sci.* 11:1213. doi: 10.3389/fpls.2020.01213

Lascoux, M., Glémin, S., and Savolainen, O. (2016). *Local Adaptation in Plants*. Chichester: John Wiley& Sons, Ltd, 1–7. doi: 10.1002/9780470015902.a0025270

Little, C. J., Wheeler, J. A., Sedlacek, J., Cortés, A. J., and Rixen, C. (2016). Small - scale drivers: the importance of nutrient availability and snowmelt timing on performance of the alpine shrub *Salix herbacea*. *Oecologia* 180, 1015–1024. doi: 10.1007/s00442-015-3394-3

López-Guzmán, G. G., Palomino-Hermosillo, Y. A., Balois-Morales, R., Bautista-Rosales, P. U., and Jiménez-Zurita, J. O. (2021). Genetic diversity of native avocado in nayarit, Mexico, determined by ISSRs. *Cienc. Tecnol. Agropecu* 22, 1–14. doi: 10.21930/rcta.vol22_num1_art:1686

López-Hernández, F., and Cortés, A. J. (2019). Last-generation genome–environment associations reveal the genetic basis of heat tolerance in common bean (*Phaseolus vulgaris* L.). *Front. Genet.* 10:954.

Loupit, G., and Cookson, S. J. (2020). Identifying molecular markers of successful graft union formation and compatibility. *Front. Plant Sci.* 11:610352. doi: 10.3389/fpls.2020.610352

Lu, X., Liu, W., Wang, T., Zhang, J., Li, X., and Zhang, W. (2020). Systemic long - distance signaling and communication between rootstock and scion in grafted vegetables. *Front. Plant Sci.* 11:460. doi: 10.3389/fpls.2020.00460

Lynch, M., and Ritland, K. (1999). Estimation of pairwise relatedness with molecular markers. *Genetics* 152, 1753–1766. doi: 10.1093/genetics/152.4.1753

Lynch, M., and Walsh, B. (1998). Genetics and analysis of quantitative traits. *Am. J. Hum. Genet.* 68, 548–549. doi: 10.1086/318209

Madam, B., Rahemi, M., Mousavi, A., and MartíNez-GóMez, P. (2011). Evaluation of the behavior of native Iranian almond species as rootstocks. *Int. J. Nuts Relat. Sci.* 2, 29–34.

Meuwissen, T. H. E., Hayes, B. J., and Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics* 157, 1819–1829. doi: 10.1093/genetics/157.4.1819

Mickelbart, M. V., and Arpaia, M. L. (2002). Rootstock influences changes in ion concentrations, growth, and photosynthesis of 'Hass' avocado trees in response to salinity. *J. Am. Soc. Hortic. Sci.* 127, 649–655. doi: 10.21273/JASHS.127.4.649

Mickelbart, M. V., Bender, G. S., Witney, G. W., Adams, C., and Arpaia, M. L. (2007). Effects of clonal rootstocks on 'Hass' avocado yield components, alternate bearing, and nutrition. *J. Hortic. Sci. Biotechnol.* 82, 460–466. doi: 10.1080/14620316.2007.11512259

Migicovsky, Z., and Myles, S. (2017). Exploiting wild relatives for genomics-assisted breeding of perennial crops. *Front. Plant Sci.* 8:460. doi: 10.3389/fpls.2017.00460

Milner, J. M., Pemberton, J. M., Brotherstone, S., and Albon, S. D. (2000). Estimating variance components and heritabilities in the wild: a case study using the 'Animal Model'. approach. *J. Evolut. Biol.* 13, 804–813. doi: 10.1046/j.1420-9101.2000.00222.x

Müller, D., Schopp, P., and Melchinger, A. E. (2017). Persistency of prediction accuracy and genetic gain in synthetic populations under recurrent genomic selection. *G3* 7, 801–811. doi: 10.1534/g3.116.036582

Muranty, H., Troggio, M., Sadok, I. B., Rifaï, M. A., Auwerkerken, A., Banchi, E., et al. (2015). Accuracy and responses of genomic selection on key traits in apple breeding. *Hortic. Res.* 2:15060. doi: 10.1038/hortres.2015.60

Neale, D. B., and Kremer, A. (2011). Forest tree genomics: growing resources and applications. *Nat. Rev. Genet.* 12, 111–122. doi: 10.1038/nrg2931

O'Brien, C., Hiti-Bandaralage, J. C., Hayward, A., and Mitter, N. (2018). *Avocado (Persea americana Mill.). In Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*. Cham.: Springer, 305–328. doi: 10.1007/978-3-319-79087-9_24

Perez, P., and De Los Campos, G. (2014). Genome-wide regression and prediction with the BGLR statistical package. *Genetics* 198, 483–495.

Picolotto, L., Fachinello, J. C., Bianchi, V. J. O., Berto, R. M., Pasa, M. D. S., and Schmitz, J. D. (2010). Yield and fruit quality of peach scion by using rootstocks propagated by air layering and seed. *Sci. Agricola* 67, 646–650. doi: 10.1590/S0103-90162010000600005

Pritchard, J. K., Stephens, M., and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959. doi: 10.1093/genetics/155.2.945

Quintero, S., Ruíz-Castañeda, W., Cubillos Jiménez, S., Marín Sánchez, B. M., Giraldo, D. P., and Vélez Acosta, L. M. (2021). Measurement technological innovation capabilities in agriculture knowledge and innovation systems. *Cienc. Tecnol. Agropecuaria* 22:e1896. doi: 10.21930/rcta.vol22_num1_art:1896

Raga, V., Bernet, P. B., Carbonell, E. A., and Asins, M. J. (2014). Inheritance of rootstock effects and their association with salt tolerance candidate genes in a progeny derived from 'Volkamer'. Lemon. *J. Am. Soc. Hortic. Sci.* 139, 518–528. doi: 10.21273/JASHS.139.5.518

Ramírez-Gil, J. G., Henao-Rojas, J. C., and Morales-Osorio, J. G. (2020a). Mitigation of the adverse effects of the El Niño (El Niño, La Niña) Southern Oscillation (ENSO) phenomenon and the Most important diseases in avocado cv. Hass crops. *Plants* 9:790. doi: 10.3390/plants9060790

Ramírez-Gil, J. G., López, J. H., and Henao-Rojas, J. C. (2020b). Causes of Hass avocado fruit rejection in preharvest, harvest, and packinghouse: economic losses and associated variables. *Agronomy* 10:8. doi: 10.3390/agronomy10010008

Rasool, A., Mansoor, S., Bhat, K. M., Hassan, G. I., Baba, T. R., Alyemeni, M. N., et al. (2020). Mechanisms underlying graft union formation and rootstock scion interaction in horticultural plants. *Front. Plant Sci* 11:590847. doi: 10.3389/fpls.2020.590847

Reeksting, B. J., Olivier, N. A., and Van Den Berg, N. (2016). Transcriptome responses of an ungrafted phytophthora root rot tolerant avocado (*Persea*

americana) rootstock to flooding and *Phytophthora cinnamomi*. *BMC Plant Biol.* 16:205. doi: 10.1186/s12870-016-0893-2

Rendón-Anaya, M., Ibarra-Laclette, E., Méndez-Bravo, A., Lan, T., Zheng, C., Carretero-Paulet, L., et al. (2019). The avocado genome informs deep angiosperm phylogeny, highlights introgressive hybridization, and reveals pathogen-influenced gene space adaptation. *PNAS* 116, 17081–17089. doi: 10.1073/pnas.1822129116

Resende, M. D. V., Resende, . M. F. R. Jr., Sansaloni, C. P., Petroli, C. D., Missiaggia, A. A., Aguiar, A. M., et al. (2012). Genomic selection for growth and wood quality in *Eucalyptus*: capturing the missing heritability and accelerating breeding for complex traits in forest trees. *New Phytol.* 194, 116–128. doi: 10.1111/j.1469-8137.2011.04038.x

Resende, R. T., Piepho, H. P., Rosa, G. J. M., Silva-Junior, O. B., Silva, F. F. E., Resende, M. D. V. D., et al. (2020). *Enviromics* in breeding: applications and perspectives on envirotypic-assisted selection. *Theor. Appl. Genet.* 134, 95–112. doi: 10.1007/s00122-020-03684-z

Reyes-Herrera, P. H., Muñoz-Baena, L., Velásquez-Zapata, V., Patiño, L., Delgado-Paz, O. A., Díaz-Diez, C. A., et al. (2020). Inheritance of Rootstock Effects in Avocado (*Persea americana* Mill.) Cv. Hass. *Front. Plant Sci.* 11:555071. doi: 10.3389/fpls.2020.555071

Robinson, H. F., Comstock, R. E., and Harvery, P. H. (1951). Genotypic and phenotypic correlation in corn and their implication in selection. *Agron. J.* 43, 282–287. doi: 10.2134/agronj1951.00021962004300060007x

Rodríguez, M., Jaramillo, J., and Orozco, J. (2009). *Colecta de Aguacates Criollos Colombianos como Base Para Iniciar Programas de Fitomejoramiento que Contribuyan a su Competitividad. In: III Congreso Latinoamericano de Aguacate*. Available online at: http://corpoaguacate.com/pdf/conferencias/pdf/Colecta%20de%20aguacates%20criollos%20colombianos.pdf (accessed January 10, 2017).

Rubinstein, M., Eshed, R., Rozen, A., Zviran, T., Kuhn, D. N., Irihimovitch, V., et al. (2019). Genetic diversity of avocado (*Persea americana* Mill.) germplasm using pooled sequencing. *BMC Genomics* 20:379. doi: 10.1186/s12864-019-5672-7

Rubio, M., Martinez-Gomez, P., Pinochet, J., and Dicenta, F. (2005). Evaluation of resistance to sharka (*Plum pox* virus) of several *Prunus* rootstocks. *Plant Breed.* 124, 67–70. doi: 10.1111/j.1439-0523.2004.01068.x

Salazar-García, S., Velasco-Cárdenas, J. J., Medina-Torres, R., and Gómez-Aguilar, J. R. (2004). Avocado selections with potential use as rootstocks I. Grafting and scion growth. *Rev. Fitotecnia Mex.* 27, 23–30.

Sánchez-González, E. I., Gutiérrez-Díez, A., and Mayek-Pérez, N. (2020). Outcrossing rate and genetic variability in Mexican race avocado. *J. Am. Soc. Hortic. Sci.* 145, 53–59. doi: 10.21273/JASHS04785-19

Sánchez-González, E. I., Gutiérrez-Soto, J. G., Olivares-Sáenz, E., Gutiérrez-Díez, A., Barrientos-Priego, A. F., and Ochoa-Ascencio, S. (2019). Screening progenies of Mexican race avocado genotypes for resistance to *Phytophthora cinnamomi* Rands. *Hortscience* 54, 809–813. doi: 10.21273/HORTSCI13552-18

Sedlacek, J., Cortés, A. J., Wheeler, J. A., Bossdorf, O., Hoch, G., Klapste, J., et al. (2016). Evolutionary potential in the alpine: trait heritabilities and performance variation of the dwarf willow *Salix herbacea* from different elevations and microhabitats. *Ecol. Evol.* 6, 3940–3952. doi: 10.1002/ece3.2171

Sharon, D., Cregan, P. B., Mhameed, S., Kusharska, M., Hillel, J., Lahav, E., et al. (1997). An integrated genetic linkage map of avocado. *Theor. Appl. Genet.* 95, 911–921. doi: 10.1007/s001220050642

Slatkin, M. (2008). Linkage disequilibrium — understanding the evolutionary past and mapping the medical future. *Nat. Rev. Genet.* 9, 477–485. doi: 10.1038/nrg2361

Smith, L. A., Dann, E. K., Pegg, K. G., Whiley, A. W., Giblin, F. R. D., and Kopittke, R. (2011). Field assessment of avocado rootstock selections for resistance to phytophthora root rot. *Aust. Plant Pathol.* 40, 39–47. doi: 10.1007/s13313-010-0011-0

Sun, W. (2012). A statistical framework for eQTL mapping using RNA-Seq data. *Biometrics* 68, 1–11. doi: 10.1111/j.1541-0420.2011.01654.x

Talavera, A., Soorni, A., Bombarely, A., Matas, A. J., and Hormaza, J. I. (2019). Genome-wide SNP discovery and genomic characterization in Avocado (*Persea americana* Mill.). *Sci. Rep.* 9:20137. doi: 10.1038/s41598-019-56526-4

Tamayo-Vélez, A. í, Bernal, J. A., and Diìaz, C. A. (2018). Composition and removal of nutrients by the harvested fruit of avocado cv. Hass in Antioquia. *Rev. Facul. Nacional Agron.* 71, 8511–8516. doi: 10.15446/rfna.v71n2.71929

Tamayo-Vélez, A. ì, and Osorio, N. W. (2018). Soil fertility improvement by litter decomposition and inoculation with the fungus *Mortierella* sp. in avocado plantations of Colombia. *Commun. Soil Sci. Plant Anal.* 49, 139–147. doi: 10.1080/00103624.2017.1417420

Tan, B., Grattapaglia, D., Martins, G. S., Ferreira, K. Z., Sundberg, B. R., and Ingvarsson, P. R. K. (2017). Evaluating the accuracy of genomic prediction of growth and wood traits in two *Eucalyptus* species and their F1 hybrids. *BMC Plant Biol.* 17:110. doi: 10.1186/s12870-017-1059-6

Tang, X., Huang, Y., Lei, J. J., Luo, H., and Zhu, X. (2019). The single-cell sequencing: new developments and medical applications. *Cell Biosci.* 9:53. doi: 10.1186/s13578-019-0314-y

Téliz, O. D. (2000). *El Aguacate y su Manejo Integrado*. México: Mundi-Prensa, D. F, 219.

Thistlethwaite, F. R., Gamal El-Dien, O., Ratcliffe, B., Klapste, J., Porth, I., Chen, C., et al. (2020). Linkage disequilibrium vs. pedigree: genomic selection prediction accuracy in conifer species. *PLoS One* 15:e0232201. doi: 10.1371/journal.pone.0232201

Thistlethwaite, F. R., Ratcliffe, B., Klápště, J., Porth, I., Chen, C., Stoehr, M. U., et al. (2019). Genomic selection of juvenile height across a single-generational gap in Douglas-fir. *Heredity* 122, 848–863. doi: 10.1038/s41437-018-0172-0

Toapanta-Gallegos, D. E., Morillo-Velastegui, L. E., and Viera-Arroyo, W. F. (2017). Molecular diagnosis of *Phytophthora cinnamomi* associated with root rot in avocado producing areas of Ecuador. *Cienc. Tecnol. Agropecu.* 18:285. doi: 10.21930/rcta.vol18_num2_art:628

Tworkoski, T., and Miller, S. (2007). Rootstock effect on growth of apple scions with different growth habits. *Sci. Hortic.* 111, 335–343. doi: 10.1016/j.scienta.2006.10.034

Wang, J., Jiang, L., and Wu, R. (2017). Plant grafting: how genetic exchange promotes vascular reconnection. *New Phytol.* 214, 56–65. doi: 10.1111/nph.14383

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., Von Wettberg, E. J. B., et al. (2016). Rootstocks: diversity, domestication, and impacts on shoot phenotypes. *Trends Plant Sci.* 21, 418–437. doi: 10.1016/j.tplants.2015.11.008

Whiley, A. W., and Wolstenholme, B. N. (1990). Carbohydrate management in avocado trees for increased production. *S. Afr. Avocado Growers Assoc. Yearb.* 13, 25–27.

Willingham, S. L., Pegg, K. G., Cooke, A. W., Coates, L. M., Langdon, P. W. B., and Dean, J. R. (2001). Rootstock influences postharvest anthracnose development in 'Hass' avocado. *Aust. J. Agric. Res.* 52, 1017–1022. doi: 10.1071/AR01015

Wilson, A. J., Réale, D., Clements, M. N., Morrissey, M. M., Postma, E., Walling, C. A., et al. (2010). An ecologist's guide to the animal model. *J. Anim. Ecol.* 79, 13–26. doi: 10.1111/j.1365-2656.2009.01639.x

Zhang, H., Yin, L., Wang, M., Yuan, X., and Liu, X. (2019). Factors affecting the accuracy of genomic selection for agricultural economic traits in maize, cattle, and pig populations. *Front. Genet.* 10:189. doi: 10.3389/fgene.2019.00189

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

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: frontiersin.org/about/contact

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