UNIVERSITY OF CALIFORNIA
RIVERSIDE

Developing Microbiome-Based Strategies for Citriculture

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Biology

by

Mengyuan Xi

December 2022

Dissertation Committee:
Dr. Philippe Rolshausen, Chairperson
Dr. Louis Santiago
Dr. Jason Stajich

The Dissertation of Mengyuan Xi is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

Acknowledgments

Foremost, I'd want to express my gratitude to my adviser, Dr. Philippe Rolshausen, for letting me work in his lab on horticulture crop research as I desired, treating me with respect when it came to my research preferences, and being so generous with his time, wisdom, and sense of humor. Dr. Louis Santiago and Dr. Jason Stajich, members of my dissertation committee, have my deepest appreciation. They are extremely knowledgeable and have supervised my research projects and given me helpful feedback on my thesis. In addition, I'd like to express my gratitude to Dr. Caroline Roper, who mentored me throughout my research on the citrus microbiome; Dr. Elizabeth Deyett, who taught me the ins and outs of laboratory work and microbiome analysis; Dr. Vanessa Ashworth, who was always there to help me with anything I needed during my daily lab sessions; and Dr. Sydney Glassman, who provided invaluable feedback on Chapter 2.

The chapter 2 of this dissertation is a reprint of the material as it appears in the following journals:

Arbuscular mycorrhizal fungal composition across US citrus orchards, management strategies, and disease severity spectrum published in Phytobiomes (2022).

The co-author Philippe Rolshausen listed in that publication directed and supervised the research which forms the basis for this dissertation.

We thank the California Citrus Research Board (Grant Numbers 5300-164 and 6100), the California Department of Food and Agriculture (Grant Number SCB16056)

and National Institute of Food and Agriculture (Grant Number 2017-70016-26053) for funding support.

In addition, I'd like to thank the members of my qualifying exam committee, Dr. Mikeal Roose, Dr. Peegy Mauk, Dr. Juan Pablo, and Dr. Milton McGiffen, for their guidance, support, and insightful comments.

I appreciate the help of everyone who has worked at the Rolshausen and Roper lab. Without them, I wouldn't be having such a great time in my Ph.D. program. As always, I appreciate the support of my loved ones, whether they are here in the United States or in China. Their spiritual support got me through every difficulty I've ever faced.

Dedication

I dedicate this dissertation to my beloved family: my husband Nan Hua, my mom Yinghua Hou and my Dad Jianchun Xi, for their unconditional love and fully support on my life decisions.

ABSTRACT OF THE DISSERTATION

Developing Microbiome-Based Strategies for Citriculture

by

Mengyuan Xi

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, December 2022
Dr. Philippe Rolshausen, Chairperson

Evolutionary selection between plants and microorganisms contributes to the system's stability. Microorganisms are beneficial to plants in promoting plant growth and alleviating biotic and abiotic stress. Using the microbiome at work provides a viable path toward establishing a more sustainable agriculture. Citrus, a vital part of California's cuisine, landscape, and economy, is threatened by an incurable and fatal plant disease, Huanglongbing (HLB). Recent advances in understanding the citrus microbiome included the composition and function of the microbiome in the different citrus bio compartments and HLB-infected citrus. However, additional work and research are necessary prior to their application in the field.

This study is a comprehensive, culture-independent microbial study of citrus that includes previously unknown plant niches, flushes, and flowers. The citrus microbiome was dominated by *Acinetobacter, Sphingomonas, Streptomyces, Actinoplanes,*

Burkholderia, *Neocosmospora*, *Cladosporium*, *Solicoccozyma*, *Mortierella*, and *Fusarium*. The fungi *Alternaria*, *Cladosporium* and *Neocosmospora* and bacteria *Actinoplanes*, *Bacillus*, *Burkholderia*, *Firmicutes* and *Sphingomonas* represented ubiquitous taxa capable of colonizing all five biocompartments analyzed (root, rhizosphere, soil, flush and flower). This research also provided insightful information about citrus AMF dynamics related to geographic location, management strategy, and Huanglongbing disease. We have found a core microbiome (*Dominikia*, *Funneliformis*, *Glomus*, *Rhizophagus*, *Sclerocystis*, *Septoglomus*) and biomarkers for healthy trees (*Glomeraceae* VTX00323 and *Dominikia* VTX00132 were significantly depleted as HLB symptoms became more severe). Finally, we have developed a bioassay to screen for potential PGPR microbes on citrus rootstock seedlings. 'Carrizo' citrange seeds were inoculated with 10 potential PGPR and all were able to colonize the host plant root/rhizosphere. Three *Bacillus* species significantly increased the seed germination rate. Two *Bacillus* species and one *Rhizobium* species significantly increased the plant height, leaf number, and shoot biomass of seedlings.

In conclusion, this study reveals the bacteriome and mycobiome within citrus, ranging from symbiotic to pathogenic, deepening our understanding of the microbe-host interaction in perennial agroecosystems and mining the microbiome for novel approaches to HLB management.

Contents

List of Figures

List of Tables

1 General Introduction

1.1 Plant Microbiome and Sustainable Agriculture

Plants provide numerous environments for the growth and multiplication of microorganisms, such as bacteria, fungus, protists, nematodes, and viruses (the plant microbiome). The plant and its associated microbiome form a holobiont, whereby evolutionary selection between plants and microorganisms contributes to the overall stability of the system (Vandenkoornhuyse et al. 2015). In recent years, culture-independent high-throughput sequencing has significantly increased the diversity of microorganisms known to inhabit plants and their surroundings (Lundberg et al. 2012; Peiffer et al. 2013; Bulgarelli et al. 2015; Coleman-Derr et al. 2016; Deyett and Rolshausen 2020; Hamonts et al. 2018; Xu et al. 2018). Genomics and multi-omics have enabled the identification and characterization of genes that regulate plant interactions with their associated microbiomes, therefore enhancing our knowledge of how microbes adapt to the plant environment (Levy et al. 2018; Cole et al. 2017).

A plant's microbiome consists of beneficial, neutral, and pathogenic microorganisms. It has been demonstrated that microbial communities linked with their hosts increase plant growth, nutrient uptake, and disease resistance (Gouda et al. 2018; Backer et al. 2018; Trivedi et al. 2016; Pieterse et al. 2014). Direct benefits of microorganisms to their host plants include transformation and translocation of essential nutrients in the soil to make them available to plants i.e. nitrogen fixation, mitigation of environmental stresses,

and protection from plant pathogens via competition, antibiosis, and the production of hydrolytic enzymes (Gouda et al. 2018; Backer et al. 2018; Trivedi et al. 2016). Indirect advantages may also result from the strengthening of a plant's resistance responses (Pieterse et al. 2014). A significant implication is that plant-associated microbial communities do not represent random assemblages but are regulated by intricate interactions between microorganisms, their plant host, and the environment (Carlström et al. 2019), despite the fact that the underlying processes are not fully understood. New insights into these complicated connections will aid in the advancement of our understanding of the evolutionary and ecological mechanisms that drive community formation and will lead translational research to enhance plant fitness and production.

Modern agriculture is mainly based on the cultivation of high-yield varieties combined with the use of agrochemicals, i.e., fertilizers and phytochemicals, for nutrient inputs and pathogen control, respectively (Kesavan and Swaminathan 2018). Because mineral fertilizers are derived from finite resources and because agrochemicals are often hazardous to the environment, i.e., soil degradation, loss of biodiversity, increased susceptibility of crops to pests/pathogens, there is a need for alternative and more sustainable agricultural practices (Tilman et al. 2002). In view of impending issues such as climate change and human population expansion, ensuring the sustainability of agriculture becomes increasingly crucial. Utilizing the microbiome at work, i.e., capitalizing on microbial features that are favorable to the host, the environment, or both, offers a viable route for the establishment of a more sustainable agriculture of the next generation (Schlaeppi and Bulgarelli 2015).

1.2 Arbuscular Mycorrhizal Fungi (AMF) and Plant Growth Promoting Rhizobacteria (PGPR)

Among the microorganisms beneficial to plants, AMF and PGPR are the two major groups that their molecular mechanisms have been thoroughly studied (Backer et al. 2018; Trivedi et al. 2016; Pieterse et al. 2014; Richardson and Simpson 2011). AMF are biotrophic organisms forming symbiotic associations with more than 70% of the land plants across a broad range of terrestrial ecosystems (Smith and Read 2008; Brundrett and Tedersoo 2018). The nature of the mutualistic symbiosis resides in a trade-off between a more efficient root acquisition of water and nutrients (especially phosphorus) via the mycorrhizal hyphal network, in exchange for photo-assimilated carbon. The outcome of this interaction often results in improved plant environmental fitness with increased tolerance to biotic and abiotic stresses (Hohmann and Messmer 2017; Chen et al. 2018). In addition, AMF improve soil structure by forming stable soil aggregates thereby limiting erosion and leaching of nutrients (Wilson et al. 2009; Chen et al. 2018). PGPR inhabit the rhizosphere, where they account for 5 to 17% of total root surface area (Gray and Smith, 2005). These microorganisms have positive effects on plant growth, seed germination, and seedling emergence (Ahmad et al., 2008). By producing plant hormones (auxins, gibberellins and cytokinin), fixing nitrogen, solubilizing inorganic phosphate, and mineralizing organic phosphate, soil microbes may boost plant growth and make these nutrients available to plants (Bhattacharyya and Jha 2012). In addition, they demonstrate synergistic and antagonistic interactions with soil bacteria and engage

in a variety of ecologically significant activities. Due to its growth-promoting effects, PGPR are regarded as an eco-friendly alternative to dangerous chemical fertilizers (Basu et al. 2021). Together, the employment of AMF and PGPR as biofertilizers constitutes a biological strategy for the intensification of agriculture in a sustainable manner.

1.3 Citrus and the Devastating Huanglongbing (HLB)

Citrus is a vital part of California cuisine, landscape, and economy. Commercially grown citrus contributes $3.3 billion in economic activity and employs more than 22,000 individuals in California (Milosavljević et al. 2021). The incurable and fatal plant disease Huanglongbing (HLB) threatens to erase this tradition from our state's history and put thousands out of work (Hodges and Spreen 2012). Since HLB was first found in 2005, citrus acreage and yield in Florida decreased by 38% and 74%, respectively. Sweet orange production dropped from 150 million boxes in 2005–2006 to 72 million boxes in 2018–2019 (Graham et al. 2020) and between 2012 and 2016 there has been a loss of $4.4 billion and 7,945 fulltime and part-time jobs (Court et al. 2018). In 2012, HLB was discovered in California in 2012 and as of March 2020, more than 2029 trees were detected as HLB-positive (Graham et al. 2020).

HLB, commonly known as citrus greening, is thought to be caused by the phloem restricted bacteria *Candidatus* Liberibacter asiaticus (*C*Las) in the U.S. Due to the absence of effective curative treatments and the lengthy incubation time, HLB management remains difficult. In order to reduce the spread of the disease, modern HLB management includes manufacture of budwood in insect-proof nurseries, frequent

examination, detection, and eradication of sick trees, as well as control of the vector Asian citrus psyllid (*Diaphorina citri*) (Zhang et al. 2014; Hu et al. 2018; Patt et al. 2018; Zhang et al. 2019; Ferrarezi et al. 2019; Gabriel et al. 2020). Other approaches such as the use of broad spectrum antimicrobials, small molecule compounds, and microorganisms that stimulate plant growth or trigger host defenses have been evaluated for HLB treatment with promising results (Munir et al. 2018; Blaustein et al. 2018). Moreover, research on the microbiome of HLB-infected citrus has yielded fresh insights into the identification of beneficial microbial communities for disease management (Blacutt et al. 2020; Ginnan et al. 2020, 2018; Xi et al. 2022). However, additional efforts and studies are required prior to their implementation in the field.

1.4 Citrus Microbiome at Work

There is a significant amount of interest in investigating the structure and function of the citrus microbiome and engineering the citrus microbiome to address a variety of problems (Zhang et al. 2021). Recent advances in understanding the citrus microbiome included the composition and function of the microbiome in the rhizosphere (Xu et al. 2018; Trivedi et al. 2010; Zhang et al. 2017; Ginnan et al. 2020), rhizoplane (Zhang et al. 2017, 2020; Blacutt et al. 2020; Riera et al. 2017, 2018), root endosphere (Ascunce et al. 2019; Bai et al. 2019; Blaustein et al. 2017; Ginnan et al. 2020; Passera et al. 2018; Trivedi et al. 2011), phyllosphere (foliar microbiome) (Bai et al. 2019; Ginnan et al. 2022, 2020; Wu et al. 2020) and core members (Zhang et al. 2021; Blaustein et al. 2017; Xu et al. 2018; Ginnan et al. 2020), and their functional traits (Xu et al. 2018). Significant

progress has been made in recent years in determining the composition and functional potential of plant-associated microbiomes (Leach et al. 2017; Xu et al. 2018; Trivedi et al. 2020). Advancements in modeling and data visualization allow us to discover keystone microorganisms that coordinate differences in the structure and function of the microbiome (Agler et al. 2016). Notably, observation to application is not a simple and direct process, and extensive efforts are required for identifying applicable inoculants from these candidate microbial groups. This is due to individuals affiliated with the same taxon in the microbiome would exhibit distinct abilities on pathogen antagonism and plant colonization (Mauchline and Malone 2017).

This research aims to (i) profile the citrus microbiome in a variety of biocompartments during the flowering stage. (ii) provide insightful information about citrus AMF dynamics related to geographic location, management strategy, and Huanglongbing disease. (iii) identify possible generalist and specialist taxa of microorganisms that could be used in agriculture. (iv) develop a growth chamber bioassay to screen for potential PGPR microbes on citrus rootstock seedlings. Taken together, this study reveals the bacteriome and mycobiome within citrus, ranging from symbiotic to pathogenic, deepening our understanding of the microbe-host interaction in perennial agroecosystems and mining the microbiome for novel approaches to HLB management.

1.5 References

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S.-T., Weigel, D., et al. 2016. Microbial hub taxa link host and abiotic factors to plant microbiome variation. PLoS Biol. 14:e1002352.

Ahmad, F., Ahmad, I., and Khan, M. S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. 163:173–181.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

Ascunce, M. S., Shin, K., Huguet-Tapia, J. C., Poudel, R., Garrett, K. A., van Bruggen, A. H. C., et al. 2019. Penicillin trunk injection affects bacterial community structure in citrus trees. Microb. Ecol. 78:457–469.

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. 2018. Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front. Plant Sci. :1473.

Bai, Y., Wang, J., Jin, L., Zhan, Z., Guan, L., Zheng, G., et al. 2019. Deciphering bacterial community variation during soil and leaf treatments with biologicals and biofertilizers to control huanglongbing in citrus trees. J. Phytopathol. 167:686–694.

Basu, A., Prasad, P., Das, S. N., Kalam, S., Sayyed, R. Z., Reddy, M. S., et al. 2021. Plant growth promoting rhizobacteria (PGPR) as green bioinoculants: recent developments, constraints, and prospects. Sustainability. 13:1140.

Belin, C., Megies, C., Hauserova, E., and Lopez-Molina, L. 2009. Abscisic acid represses growth of the Arabidopsis embryonic axis after germination by enhancing auxin signaling. Plant Cell. 21:2253–2268.

Benizri, E., Baudoin, E., and Guckert, A. 2001. Root colonization by inoculated plant growth-promoting rhizobacteria. Biocontrol Sci. Technol. 11:557–574.

Beyer, M., and Diekmann, H. 1985. The chitinase system ofStreptomyces sp. ATCC 11238 and its significance for fungal cell wall degradation. Appl. Microbiol. Biotechnol. 23:140–146.

Bhattacharyya, P. N., and Jha, D. K. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28:1327–1350.

Bishnoi, U. 2018. Agriculture and the dark side of chemical fertilizers. Environ. Anal. Ecol. Stud. 3.

Blacutt, A., Ginnan, N., Dang, T., Bodaghi, S., Vidalakis, G., Ruegger, P., et al. 2020. An in vitro pipeline for screening and selection of citrus-Associated microbiota with potential anti-"Candidatus liberibacter asiaticus" properties. Appl. Environ. Microbiol. 86:1–18.

Blaustein, R. A., Lorca, G. L., Meyer, J. L., Gonzalez, C. F., and Teplitski, M. 2017. Defining the core citrus leaf- and root-associated microbiota: Factors associated with community structure and implications for managing huanglongbing (citrus greening) disease. Appl. Environ. Microbiol. 83.

Blaustein, R. A., Lorca, G. L., and Teplitski, M. 2018. Challenges for managing Candidatus Liberibacter spp.(Huanglongbing disease pathogen): Current control measures and future directions. Phytopathology. 108:424–435.

Brundrett, M. C., and Tedersoo, L. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 220:1108–1115.

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., Pan, Y., et al. 2015. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe. 17:392–403.

Carlström, C. I., Field, C. M., Bortfeld-Miller, M., Müller, B., Sunagawa, S., and Vorholt, J. A. 2019. Synthetic microbiota reveal priority effects and keystone strains in the Arabidopsis phyllosphere. Nat. Ecol. Evol. 3:1445–1454.

Chelius, M. K., and Triplett, E. W. 2001. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. :252–263.

Chen, M., Arato, M., Borghi, L., Nouri, E., and Reinhardt, D. 2018. Beneficial services of arbuscular mycorrhizal fungi–from ecology to application. Front. Plant Sci. 9:1270.

Cole, B. J., Feltcher, M. E., Waters, R. J., Wetmore, K. M., Mucyn, T. S., Ryan, E. M., et al. 2017. Genome-wide identification of bacterial plant colonization genes. PLoS Biol. 15:e2002860.

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., et al. 2016. Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. New Phytol. 209:798–811.

Court, C. D., Hodges, A. W., Rahmani, M., and Spreen, T. 2018. Economic Contributions of the Florida Citrus Industry in 2015-16: FE1021, 7/2017. EDIS. 2018.

Damayanti, T. A., Pardede, H., and Mubarik, N. R. 2007. Utilization of root-colonizing bacteria to protect hot-pepper against Tobacco Mosaic Tobamovirus. HAYATI J. Biosci. 14:105–109.

Davison, J., Moora, M., Öpik, M., Adholeya, A., Ainsaar, L., Bâ, A., et al. 2015. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. Science (80-.). 349.

Davison, J., Moora, M., Semchenko, M., Adenan, S. B., Ahmed, T., Akhmetzhanova, A. A., et al. 2021. Temperature and pH define the realised niche space of arbuscular mycorrhizal fungi. New Phytol. 231:763–776.

Deyett, E., and Rolshausen, P. E. 2020. Endophytic microbial assemblage in grapevine. FEMS Microbiol. Ecol. 96.

Esitken, A., Karlidag, H., Ercisli, S., Turan, M., and Sahin, F. 2003. The effect of spraying a growth promoting bacterium on the yield, growth and nutrient element composition of leaves of apricot (Prunus armeniaca L. cv. Hacihaliloglu). Aust. J. Agric. Res. 54:377–380.

Ferrarezi, R. S., Qureshi, J. A., Wright, A. L., Ritenour, M. A., and Macan, N. P. F. 2019. Citrus production under screen as a strategy to protect grapefruit trees from Huanglongbing disease. Front. Plant Sci. 10:1598.

Franco-Correa, M., Quintana, A., Duque, C., Suarez, C., Rodríguez, M. X., and Barea, J.-M. 2010. Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. Appl. Soil Ecol. 45:209–217.

Gabriel, D., Gottwald, T. R., Lopes, S. A., and Wulff, N. A. 2020. Bacterial pathogens of citrus: Citrus canker, citrus variegated chlorosis and Huanglongbing. In *The genus citrus*, Elsevier, p. 371–389.

Gao, C., Montoya, L., Xu, L., Madera, M., Hollingsworth, J., Purdom, E., et al. 2019. Strong succession in arbuscular mycorrhizal fungal communities. ISME J. 13.

Genin, S., and Denny, T. P. 2012. Pathogenomics of the Ralstonia solanacearum species complex. Annu. Rev. Phytopathol. 50:67–89.

Giassi, V., Kiritani, C., and Kupper, K. C. 2016. Bacteria as growth-promoting agents for citrus rootstocks. Microbiol. Res. 190:46–54.

Ginnan, N. A., De Anda, N. I., Campos Freitas Vieira, F., Rolshausen, P. E., and Roper, M. C. 2022. Microbial Turnover and Dispersal Events Occur in Synchrony with Plant Phenology in the Perennial Evergreen Tree Crop Citrus sinensis. MBio. :e00343-22.

Ginnan, N. A., Dang, T., Bodaghi, S., Ruegger, P. M., McCollum, G., England, G., et al. 2020. Disease-induced microbial shifts in citrus indicate microbiome-derived responses to huanglongbing across the disease severity spectrum. Phytobiomes J. 4:375–387.

Ginnan, N. A., Dang, T., Bodaghi, S., Ruegger, P. M., Peacock, B. B., McCollum, G., et al. 2018. Bacterial and Fungal Next Generation Sequencing Datasets and Metadata from Citrus Infected with 'Candidatus Liberibacter asiaticus.' Phytobiomes J. 2:64–70.

Goswami, D., Dhandhukia, P., Patel, P., and Thakker, J. N. 2014. Screening of PGPR from saline desert of Kutch: growth promotion in Arachis hypogea by Bacillus licheniformis A2. Microbiol. Res. 169:66–75.

Goswami, D., Thakker, J. N., and Dhandhukia, P. C. 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric. 2:1127500.

Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H.-S., and Patra, J. K. 2018. Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiol. Res. 206:131–140.

Graham, J., Gottwald, T., and Setamou, M. 2020. Status of Huanglongbing (HLB) outbreaks in Florida, California and Texas. Trop. Plant Pathol. 45:265–278.

Graham, J. H., Duncan, L. W., and Eissenstat, D. M. 1997. Carbohydrate allocation patterns in citrus genotypes as affected by phosphorus nutrition, mycorrhizal colonization and mycorrhizal dependency. New Phytol. 135:335–343.

Groot, S. P. C., and Karssen, C. M. 1987. Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. Planta. 171:525–531.

Gutiérrez-Mañero, F. J., Ramos-Solano, B., Probanza, A. n, Mehouachi, J., R. Tadeo, F., and Talon, M. 2001. The plant-growth-promoting rhizobacteria Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins. Physiol. Plant. 111:206–211.

Halder, A. K., Mishra, A. K., Bhattacharyya, P., and Chakrabartty, P. K. 1990. Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. J. Gen. Appl. Microbiol. 36:81–92.

Hamonts, K., Trivedi, P., Garg, A., Janitz, C., Grinyer, J., Holford, P., et al. 2018. Field study reveals core plant microbiota and relative importance of their drivers. Environ. Microbiol. 20:124–140.

Hervé, M. 2018. RVAideMemoire: testing and plotting procedures for biostatistics. R Packag. version 0.9-69. 3.

Hodges, A. W., and Spreen, T. H. 2012. Economic impacts of citrus greening (HLB) in Florida, 2006/07–2010/11. EDIS. 2012.

Hohmann, P., and Messmer, M. M. 2017. Breeding for mycorrhizal symbiosis: focus on disease resistance. Euphytica. 213:113.

Hu, J., Jiang, J., and Wang, N. 2018. Control of citrus Huanglongbing via trunk injection of plant defense activators and antibiotics. Phytopathology. 108:186–195.

Johnson, N. C., Graham, J. H., and Smith, F. A. 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. New Phytol. 135:575–585.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., and Pozo, M. J. 2012. Mycorrhiza-induced resistance and priming of plant defenses. J. Chem. Ecol. 38:651–664.

Kesavan, P. C., and Swaminathan, M. S. 2018. Modern technologies for sustainable food and nutrition security. Curr. Sci. 115:1876–1883.

Lane, D. J. 1. 1991. 16S/23S rRNA sequencing. Nucleic acid Tech. Bact. Syst. :115–175.

Leach, J. E., Triplett, L. R., Argueso, C. T., and Trivedi, P. 2017. Communication in the phytobiome. Cell. 169:587–596.

Levy, A., Salas Gonzalez, I., Mittelviefhaus, M., Clingenpeel, S., Herrera Paredes, S., Miao, J., et al. 2018. Genomic features of bacterial adaptation to plants. Nat. Genet. 50:138–150.

Li, S., Wu, F., Duan, Y., Singerman, A., and Guan, Z. 2020. Citrus greening: Management strategies and their economic impact. HortScience. 55:604–612.

Liu, J., Ma, K., Ciais, P., and Polasky, S. 2016. Reducing human nitrogen use for food production. Sci. Rep. 6:1–14.

Van Loon, L. C. 2007. Plant responses to plant growth-promoting rhizobacteria. New Perspect. approaches plant growth-promoting Rhizobacteria Res. :243–254.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. 2012. Defining the core Arabidopsis thaliana root microbiome. Nature. 488:86–90.

Marcos Filho, J. 2015. Seed vigor testing: an overview of the past, present and future perspective. Sci. Agric. 72:363–374.

Mauchline, T. H., and Malone, J. G. 2017. Life in earth–the root microbiome to the rescue? Curr. Opin. Microbiol. 37:23–28.

McCarthy, M. C., and Enquist, B. J. 2007. Consistency between an allometric approach and optimal partitioning theory in global patterns of plant biomass allocation. Funct. Ecol. :713–720.

Milosavljević, I., Morgan, D. J. W., Massie, R. E., and Hoddle, M. S. 2021. Density dependent mortality, climate, and Argentine ants affect population dynamics of an invasive citrus pest, Diaphorina citri, and its specialist parasitoid, Tamarixia radiata, in Southern California, USA. Biol. Control. 159:104627.

Munir, S., He, P., Wu, Y., He, P., Khan, S., Huang, M., et al. 2018. Huanglongbing control: perhaps the end of the beginning. Microb. Ecol. 76:192–204.

Munne-Bosch, S., Onate, M., Oliveira, P. G., and Garcia, Q. S. 2011. Changes in phytohormones and oxidative stress markers in buried seeds of Vellozia alata. Flora-Morphology, Distrib. Funct. Ecol. Plants. 206:704–711.

Mushtaq, S., Shafiq, M., Ashraf, T., Haider, M. S., Ashfaq, M., and Ali, M. 2019. Characterization of plant growth promoting activities of bacterial endophytes and their antibacterial potential isolated from citrus. JAPS J. Anim. Plant Sci. 29.

Orbović, V., Dutt, M., and Grosser, J. W. 2013. Evaluation of the germination potential of citrus seeds during the harvesting season. HortScience. 48:1197–1199.

Ortíz-Castro, R., Contreras-Cornejo, H. A., Macías-Rodríguez, L., and López-Bucio, J. 2009. The role of microbial signals in plant growth and development. Plant Signal. Behav. 4:701–712.

Passera, A., Alizadeh, H., Azadvar, M., Quaglino, F., Alizadeh, A., Casati, P., et al. 2018. Studies of microbiota dynamics reveals association of “Candidatus Liberibacter asiaticus” infection with citrus (Citrus sinensis) decline in south of Iran. Int. J. Mol. Sci. 19:1817.

Patt, J. M., Meikle, W. G., Niedz, R. P., and Woods, D. 2018. Synthetic ligands of olfactory binding proteins modulate aggregation response of Asian citrus psyllid in the presence of host-plant volatiles. Front. Plant Sci. 9:1891.

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. 110:6548–6553.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. 2014. Induced systemic resistance by beneficial microbes. Annu. Rev. Phytopathol. 52:347–375.

Pingali, P. L. 2012. Green revolution: impacts, limits, and the path ahead. Proc. Natl. Acad. Sci. 109:12302–12308.

Qi, Y., Wei, W., Chen, C., and Chen, L. 2019. Plant root-shoot biomass allocation over diverse biomes: A global synthesis. Glob. Ecol. Conserv. 18:e00606.

Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C., et al. 2012. Seed germination and vigor. Annu. Rev. Plant Biol. 63:2012.

Richardson, A. E., and Simpson, R. J. 2011. Soil microorganisms mediating phosphorus availability update on microbial phosphorus. Plant Physiol. 156:989–996.

Riera, N., Handique, U., Zhang, Y., Dewdney, M. M., and Wang, N. 2017. Characterization of antimicrobial-producing beneficial bacteria isolated from huanglongbing escape citrus trees. Front. Microbiol. 8:2415.

Riera, N., Wang, H., Li, Y., Li, J., Pelz-Stelinski, K., and Wang, N. 2018. Induced systemic resistance against citrus canker disease by rhizobacteria. Phytopathology. 108:1038–1045.

Rodríguez, H., and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. 17:319–339.

Schlaeppi, K., and Bulgarelli, D. 2015. The plant microbiome at work. Mol. Plant-Microbe Interact. 28:212–217.

Smith, S., and Read, D. 2008. *Mycorrhizal Symbiosis*.

Song, F., Pan, Z., Bai, F., An, J., Liu, J., Guo, W., et al. 2015. The scion/rootstock genotypes and habitats affect arbuscular mycorrhizal fungal community in citrus. Front. Microbiol. 6.

Steenhoudt, O., and Vanderleyden, J. 2000. Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24:487–506.

Subbiah, V., and Reddy, K. J. 2010. Interactions between ethylene, abscisic acid and cytokinin during germination and seedling establishment in Arabidopsis. J. Biosci. 35:451–458.

Sun, T., and Gubler, F. 2004. Molecular mechanism of gibberellin signaling in plants. Annu. Rev. Plant Biol. 55:197–223.

Swain, M. R., Naskar, S. K., and Ray, R. C. 2007. Indole-3-acetic acid production and effect on sprouting of yam (Dioscorea rotundata L.) minisetts by Bacillus subtilis isolated from culturable cowdung microflora. Polish J. Microbiol. 56:103.

Thokchom, E., Kalita, M. C., and Talukdar, N. C. 2014. Isolation, screening, characterization, and selection of superior rhizobacterial strains as bioinoculants for seedling emergence and growth promotion of Mandarin orange (Citrus reticulata Blanco). Can. J. Microbiol. 60:85–92.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. 2002. Agricultural sustainability and intensive production practices. Nature. 418:671–677.

Trivedi, P., Duan, Y., and Wang, N. 2010. Huanglongbing, a systemic disease, restructures the bacterial community associated with citrus roots. Appl. Environ. Microbiol. 76:3427–3436.

Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., and Singh, B. K. 2020. Plant–microbiome interactions: from community assembly to plant health. Nat. Rev. Microbiol. 18:607–621.

Trivedi, P., Pandey, A., and Palni, L. M. S. 2005. Carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cooler regions. World J. Microbiol. Biotechnol. 21:941–945.

Trivedi, P., Spann, T., and Wang, N. 2011. Isolation and characterization of beneficial bacteria associated with citrus roots in Florida. Microb. Ecol. 62:324–336.

Trivedi, P., Trivedi, C., Grinyer, J., Anderson, I. C., and Singh, B. K. 2016. Harnessing host-vector microbiome for sustainable plant disease management of phloem-limited bacteria. Front. Plant Sci. 7:1423.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. 2015. The importance of the microbiome of the plant holobiont. New Phytol. 206:1196–1206.

Velten, S., Leventon, J., Jager, N., and Newig, J. 2015. What is sustainable agriculture? A systematic review. Sustainability. 7:7833–7865.

Wilson, G. W. T., Rice, C. W., Rillig, M. C., Springer, A., and Hartnett, D. C. 2009. Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecol. Lett. 12:452–461.

Wu, Y., Qu, M., Pu, X., Lin, J., and Shu, B. 2020. Distinct microbial communities among different tissues of citrus tree Citrus reticulata cv. Chachiensis. Sci. Rep. 10.

Xi, M., Deyett, E., Ginnan, N., Ashworth, V. E. T. M., Dang, T., Bodaghi, S., et al. 2022. Arbuscular mycorrhizal fungal composition across US citrus orchards, management strategies, and disease severity spectrum. bioRxiv.

Xu, J., Zhang, Y., Zhang, P., Trivedi, P., Riera, N., Wang, Y., et al. 2018. The structure and function of the global citrus rhizosphere microbiome. Nat. Commun. 9.

Yuan, W. M., and Crawford, D. L. 1995. Characterization of Streptomyces lydicus WYEC108 as a potential biocontrol agent against fungal root and seed rots. Appl. Environ. Microbiol. 61:3119–3128.

Zahir, Z. A., Arshad, M., and Frankenberger, W. T. 2004. Plant growth promoting. Adv. Agron. 81:97.

Zhang, M., Guo, Y., Powell, C. A., Doud, M. S., Yang, C., and Duan, Y. 2014. Effective antibiotics against 'Candidatus Liberibacter asiaticus' in HLB-affected citrus plants identified via the graft-based evaluation. PLoS One. 9:e111032.

Zhang, M., Yang, C., Powell, C. A., Avery, P. B., Wang, J., Huang, Y., et al. 2019. Field evaluation of integrated management for mitigating citrus huanglongbing in Florida. Front. Plant Sci. 9:1890.

Zhang, Y., Trivedi, P., Xu, J., Caroline Roper, M., and Wang, N. 2021. The Citrus Microbiome: From Structure and Function to Microbiome Engineering and beyond. Phytobiomes J. 5:249–262.

Zhang, Y., Xu, J., Riera, N., Jin, T., Li, J., and Wang, N. 2017. Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. Microbiome. 5.

Zhang, Y., Xu, J., Wang, E., and Wang, N. 2020. Mechanisms underlying the rhizosphere-to-rhizoplane enrichment of cellvibrio unveiled by genome-centric metagenomics and metatranscriptomics. Microorganisms. 8:583.

2 Microbiome Diversity, Composition and Assembly in California Citrus

2.1 Introduction

Plant microbiomes have been shown to benefit plants by priming the immune system and protecting from them diseases, facilitating the nutrient acquisition, and overall enhancing health and increasing yield. Since the green revolution, modern agriculture is mainly based on the cultivation of high-yield varieties combined with the use of agrochemicals. Mineral fertilizers are derived from finite resources and agrochemicals are often hazardous to the environment and lead to soil degradation, loss of biodiversity, increased susceptibility of crops to pests/pathogens, and negative environmental impacts which, together, have significant consequences for human health and food security (Tilman et al., 2002). Ensuring the sustainability of agriculture has become critical considering future challenges such as climate change or the rapid growth of the human population. Taking advantage of the microbiome at work, i.e., the capitalization on microbial traits that are beneficial to the host or the environment or both, presents a promising avenue for the development of a more sustainable next-generation agriculture (Schlaeppi and Bulgarelli, 2015).

Commonly occurring organisms across similar microbiomes form a core microbial community that is hypothesized to play critical roles in ecosystem functioning within that type of microbial habitat (Shade and Handelsman, 2012; Gopal et al., 2013). While many deep sequencing studies have shown that plant microbiomes are made up of thousands of

microbial taxa, only a few taxa typically predominate in the larger community (Sagaram et al., 2009; Gottel et al., 2011; Weinert et al., 2011; Bodenhausen et al., 2013; Peiffer et al., 2013). Even in a variety of experimental settings, some of the highly abundant taxa in these studies are noticeably conserved across the microbiomes of related plant species. This implies that a core microbial community consistently associates with specific hosts at different spatial and temporal scales. However, it is known that the composition of the plant microbiota is influenced by a number of biotic (such as the stage of plant development and phytopathogens) and abiotic (such as soil type, climate, and season) factors (Redford and Fierer, 2009; Bulgarelli et al., 2012; Lundberg et al., 2012; Rastogi et al., 2012). There is still much to learn about the composition of the core microbiome community and its significance for plant health, given that only a few studies have identified the key players in plant-associated microbial communities (Bulgarelli et al., 2012; Lundberg et al., 2012; Rastogi et al., 2012).

Citrus is one of the most important perennial fruit crops in the world. Being a good source of vitamins, fiber, and minerals, it is commended for its nutritional qualities and advantages for human health. Citrus is also a major contributor to the economic value of the agricultural sector. It accounts for 16% of the total value of U.S. fruit production (Li et al., 2020) with California representing 80% of the nation's fresh fruit market with an annual value of 2.3 billion dollars (https://www.cdfa.ca.gov/Statistics/). There has been tremendous interest in exploring the structure and function of the citrus phyllosphere and rhizosphere microbiomes and engineering its assembly to address current challenges (Zhang et al., 2021; Ginnan et al., 2022). The root microbiome serves a variety of

essential roles, including aiding in nutrient uptake, promoting plant growth by producing plant hormones, cycling carbon and nutrients, maintaining soil properties, and safeguarding the host from pathogens. In citrus, research on the rhizosphere microbiome has been mostly studied in the context of huanglongbing disease (HLB) (Blaustein et al., 2017; Xu et al., 2018; Ginnan et al., 2020; Wu et al., 2020). Data showed that microbial community structures associated with citrus are stable in mature healthy groves (Ginnan et al., 2022), but when trees become affected by HLB, it creates a microbial dysbiosis with depletion and enrichment patterns for key signature taxa (Ginnan et al., 2020). However, despite our better understanding of the importance of root health on disease management, tree health, and productivity, gaps remain to develop specific guidelines for long-term disease management.

HLB or citrus greening is considered the most serious problem for citrus worldwide (Council, 2010). HLB is caused by an uncultivable Gram-negative phloem-limited bacteria belonging to the *Candidatus* Liberibacter species (i.e., *Ca*. L. asiaticus, *C*las; *Ca*. L. africanus *C*laf; and *Ca*. L. americanus, *C*Lam), which are transmitted from infected to healthy plants by citrus psyllids (Bové, 2006). The highest pathogen concentrations can be found in the midribs of flush (Chiyaka et al., 2012). A flush shoot may be defined as a new shoot growth with immature leaves but can range from as small as newly breaking buds of just feather flush to fully elongated shoots with expanded, tender leaves. In California and Mediterranean climates, the flush is produced twice annually in relatively well-defined cycles, one related to plant growth in summer-autumn, and one related to flowering and fruiting in spring. The timing of flush development is genetically and

environmentally governed, by temperature, photoperiod, solar radiation, and rainfall (Moss, 1969, 1976; Olesen et al., 2013). The most critical of these for fruit production is the spring leaf flush since it coincides with both flowering and early fruit development. However, the microbial composition of the citrus flush has to our knowledge not been elucidated, despite the fact that this tissue is at the forefront of the infection in the HLB pathosystem and despite its importance on tree vegetative growth. Profiling the citrus flush microbiome could identify potential beneficial organisms that are inhibitory to *C*las or provide the host with environmental fitness and horticultural advantage.

Similar to the flush, the study of flower microbiome has surprisingly received little attention despite its direct role in fruit production. Research indicated that soil and rhizosphere microbiomes can drive changes in the host phenological traits including the flowering period (Lau and Lennon, 2011; Lu et al., 2018), but the role of the flower microbiome on the host phenology is unknown. Flowering is the most important determinant of yield and quality of citrus fruit production (Stander, 2015). In citrus, flowering time and intensity depend on the species, the tree age, and the climatic conditions (Lau and Lennon, 2011; Agustí et al., 2020). In California's Central Valley, citrus flowers are a significant source of nectar related to honey production. Particularly, flowers provide ephemeral but unique nutrient-rich and protective habitats for microorganisms and the microbial make-up of flowers may affect disease outcome and in turn fruit yield. For example, the growth of *Streptomyces* in strawberry flowers reduces the growth of the pathogenic fungus *Botrytis* and, in honeybees, reduces mortality from the entomopathogens *Paenibacillus larvae* and *Serratia marcescens* (Kim et al., 2019).

The understanding of the reproductive microbiome function on flowering may hold the key to enhance productivity in agroecosystems.

The objective of this study was to fill in the knowledge gap about root microbial assemblage in citrus to better identify key microbes recruited by the host that may harbor beneficial properties, increase the host environmental fitness, and support tree health. In addition, our goal was to profile the microbiome of the flower and the flush, two young tissues that had not been extensively studied despite their critical importance in vegetative and reproductive cycles. Here, we provide a microbial map of five distinct compartments (bulk soil, rhizosphere, root endosphere, flush, and flower) of citrus from a single orchard over a two-year period and discuss in what capacity the information acquired with this research may help citrus production.

2.2 Materials and Methods

Plant Sampling and Processing

The plant materials were collected from the citrus cv. ‘Tango’ on ‘Carrizo’ rootstock (*Citrus sinensis (L.) Osbeck x Poncirus trifoliata L.*) at the Lindcove Research & Extension Center, California. The citrus was planted on 6/1/2011 and all samples were collected at the flower initiation stage on 4/72021 and 3/28/2022. Flower, flush (the new foliar growth between bud break and shoot expansion), root, and rhizosphere samples were collected from 12 trees in the citrus grove. Flush and flower samples were collected from the four quadrants and pooled. Feeder roots were sampled from two sides of the tree

approximately 0.5 m away from the base of the trunk. Five bulk soil samples were also collected each year across the citrus grove. Gloves were changed and clippers and shovels were sterilized with 30% household bleach between each sampled tree. All samples were immediately placed on ice in a cooler for transit to the laboratory and were frozen. All samples were processed within 24 h. Root and rhizosphere samples were processed as described by (Lundberg et al., 2012). Briefly, roots were placed in a sterile 50-mL conical tube with 25 mL of PBS with 200-μL L−1 Silwet R L-77 surfactant. Samples were vortexed at maxim speed for 15 s. Roots were then transferred to a clean 50-mL conical tube with 25 mL of PBS. The first tube was centrifuged at 3200 g for 15 min and the aqueous layer was removed. The pellet was retained as the rhizosphere fraction. The roots continued to be vortexed and were moved to a clean PBS tube until PBS remained clear after vortexing. Roots were then sonicated using a Branson Sonifier 450 at a low frequency for 5min (five 30 s bursts followed by 30 s breaks). Roots were then stored at−70◦C for further processing. Flowers, flushes, and roots were then lyophilized in the FreeZone 2.5-L benchtop freeze dry system (Labconco, Kansas City, USA) for 72 h. Specifically, flower and flush samples were not surface sterilized; thus, our aboveground microbial next-generation sequencing datasets included both epiphytes and endophytes. Samples were then ground to a powder using the MM300 grinder (Retsch, Haan, Germany) in a 35-mL stainless steel grinding jar with 20-mm stainless steel balls at 25 oscillations per second in 30-s increments until the sample was fully pulverized.

Microbiome Library Preparation

DNA was extracted from all samples using the ZymoBIOMICS DNA miniprep kit per the manufacturer's protocol, using 100 mg of dried tissue or 250 mg of the wet rhizosphere (Zymo Research, Irvine, USA). DNA was assessed for quality and quantity using the Qubit 4 Fluorometer with the Qubit dsDNA HS Assay (Thermo Fisher Scientific Inc., MA, USA). Both bacterial 16S–V4 and fungal ITS rRNA regions were amplified from all samples using the Earth Microbiome protocol and primers (http://www.earthmicrobiome.org/). Briefly, primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) were used for bacterial microbiomes, and ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) for fungal ITS amplification (Caporaso et al., 2010). PCR reactions of 25 µL contained 10 µL of Phusion hot start flex 2× master mix, 0.5 µL of each primer (10 µm), and 2 µL of DNA. In bacterial above-ground tissue (flower and flush), universal pPNA and mPNA clamps were added at a starting concentration of 1.25 µL (5 µm). These clamps were designed to reduce the amplification of host chloroplasts and mitochondria while having no effect on bacterial amplification (Fitzpatrick et al., 2018b). Negative control was added to each PCR to ensure barcodes and master mix were not contaminated. Successful amplification was verified on a 1% agarose gel and DNA was quantified using the NanoDrop 2000 Spectrophotometers (Thermo Fisher Scientific Inc., MA, USA). 1200 ng of each sample in a library were combined into an Eppendorf tube and cleaned using the AMPure XP PCR purification system (Beckman Coulter, Brea, USA) per the manufacturer's protocol. The final

concentration of libraries was determined using both qPCR and bioanalyzer before being sequenced on the MiSeq instrument (Illumina, San Diego, USA) using Miseq run (2 × 300 paired-end) for fungal reads and Miseq runs (2 × 250 paired-end) for bacterial microbiome at the UC Riverside Genomics Core facility.

Computational Analysis

The R v4.1.1 (R Core Team, 2021) was used to perform all computational analyses. Most processing for the reads was done in DADA2 v 1.16.0 (Callahan et al., 2016) including further quality control sequencing filtering, dereplication, chimera identification, merging paired-end reads, and construction of sequence tables. Taxonomy identification was assigned using the SILVA SSU r138.1 reference database for bacterial taxa and Unite database v 10.5.2021 for fungal taxa.

Phyloseq v 1.36.0 (McMurdie and Holmes, 2013) and ggplot2 v3.3.5 packages (Wickham 2016) were used for much of the graphical and statistical analyses of the data. Unidentified microbes at the kingdom or phylum level or microbes that occurred less than two times within all 24 trees (12 tree samples per year) were removed from the full dataset.

The bacterial dataset totaled 106 samples (24 flowers, 24 flushes, 24 rhizospheres, 24 roots, and 5 soil samples) and the fungal dataset totaled 104 samples (23 flowers, 24 flushes, 24 rhizospheres, 23 roots, and 5 soil samples) after filtering out poor quality reads, chloroplast, mitochondria, taxa with unidentified phyla. After removal of

singletons and doubletons, the total ASVs were of 10 483 (soil = 4395; rhizosphere = 7635; root = 1997; flush = 129; flower = 128) and 5155 (soil = 707; rhizosphere = 2964; root = 1333; flush = 860; flower = 905) for the bacterial and fungal datasets, respectively.

Shannon diversity index was used as a metric of taxa diversity within the communities. Kruskal–Wallis and pairwise Wilcoxon rank sum tests were run to verify statistical differences among groups. Phylum bar charts and Genus bar charts were constructed by aggregating taxa at the Phylum level and Genus level, respectively. Samples were also constructed by tissue compartments and transformed to relative abundance. Bray–Curtis dissimilarity was used to calculate the compositional similarities between samples and was visualized with NMDS (Non-metric MultiDimenstional Scaling) plots using the Vegan package v 2.5-7. To determine the statistical significance of beta diversity, Adonis tests were run. Venn diagrams were created using UpSetR v 1.4.0 by transforming to relative abundance and filtering taxa to those that occur greater than 0.1% and are prevalent in at least two samples of that tissue type. For prevalent Venn diagrams, data was aggregated by genus and transformed to relative abundance. Taxa were denoted as prevalent in each biocompartment. Graphs were generated using VennDiagram v1.6.20. For concentric pie charts representing the core microbiome, data were aggregated to the ASV or genus level and transformed to relative abundance. ASVs/genera were filtered based on the core microbiome as previously defined. To find microbes associated with a biocompartment and above- and belowground sections, DeSeq2 v 1.30.1 was utilized and visualized using Pheatmap v1.0.12. Genera were filtered by relative abundance, P value, and log2 fold change, keeping only genera

occurring at ≥ 1% relative abundance of the whole dataset with $P < 0.01$ and having a log2 fold change >5 or <−5. Heat maps represent the relative abundance of the data.

2.3 Results

Shannon diversity index indicated that the richness of plant bacteriome and mycobiome shared a similar distribution pattern (Fig.1). However, the bacteriome richness was higher in the below-ground samples (soil, rhizosphere, root) than in the mycobiome, while the opposite was true for the above ground tissues (flush/flower). Overall, the rhizosphere displayed a significantly higher abundance of microbiome among all plant tissue types in both bacterial and fungal groups ($P < 0.001$ [pairwise Wilcox]), whereas the soil microbiome was only significantly lower than the rhizosphere in the fungal group ($P < 0.05$ [pairwise Wilcox]) but no difference in the bacterial group ($P = 0.44$ [pairwise Wilcox]). In the bacterial group, all below-ground samples show a significantly higher Shannon diversity index as compared to the above-ground samples ($P < 0.001$ [pairwise Wilcox]). In contrast, the root microbiome displayed significantly lower fungal community richness of all three tissue types ($P < 0.001$ [pairwise Wilcox]) but show no difference with soil fungal diversity ($P = 0.25$ [pairwise Wilcox]). Flower and flush microbiome richness levels were similar to each other in both groups. Year show effect on Shannon diversity index in fungal microbiome ($P < 0.05$ [pairwise Wilcox]) but not in bacteriome ($P = 0.23$ [pairwise Wilcox]).

Bray–Curtis beta-diversity metrics with NMDS were used to visualize how compartments impacted fungal and bacterial community composition (Fig. 2). Our data

showed distinct clustering between above- and below-ground in both bacterial and fungal communities ($P < 0.001$ [Adonis]). Within belowground samples, clear clustering was measured among soil, rhizosphere, and root in both bacterial and fungal groups. Within aboveground samples, flush and flower showed overlapping patterns in bacterial year-2 data and all fungal data. Year also had a significant effect ($P < 0.001$ [Adonis] in the bacterial group; $P < 0.05$ [Adonis] in the fungal group) in the clustering pattern, especially a big shift in the bacterial flower and flush datasets.

Proteobacteria and *Ascomycota* were the most abundant phyla within the entire dataset representing on average 47.7% and 81.6% of all taxa, respectively (Fig. 3). Phyla *Basidiomycota* and *Actinobacteria* were also important phyla as they occurred in greater than 10% on average across the entire datasets. Several phyla with a relatively great abundance (greater than 5%) were limited to belowground or aboveground. For example, *Glomeromycota*, *Mortierellomycota*, *Acidobacteria*, *Gemmatimonadota*, and *Verrucomicrobiota* were mainly found in soil, root, and rhizosphere, whereas Cyanobacteria was only found in flower and flush samples. Although the most abundant phyla were the same in each tissue at the phylum level, they were different at the genus level, with the main difference between aboveground and belowground. In the fungal group, the most abundant genus in belowground samples was *Neocosmospora* (36.4%), while in aboveground *Cladosporium* was dominant (60.4%). In the bacterial group, *Acinetobacter* was the most abundant genus in aboveground samples (38.7%), and the belowground samples were more diverse, with no dominant genus.

We used DeSeq2 analyses to indicate enrichment/rarefaction patterns of taxa along the soil, rhizosphere root continuum, and signature microbial taxa for the three plant compartments, root, flush, and flower. We focused our analysis on the most prevalent and abundant with a relative abundance of >1%. (Fig.4). Our results indicated a root enrichment of several bacterial genera from the soil to the rhizosphere but only a few of these were capable of further entering the root including *Actinoplanes, Burkholderia, Muclilagnibacter*, and *Rhizobium* and fungi *Giberrella, Glomus, Neocosmospora, Rhizophagus,* and *Setophaeosphaeria*. Several bacterial and fungal taxa were clearly root-specific and not found to include *Bradyrhizobium Cupriavidus* and *Rhizobium* and fungi *Gibberella, Glomus, Neocosmospora, Rhizophagus,* and *Setophaeosphaeria.* In contrast, the bacteria *Acinetobacter, Aquabacterium, Gilliamella, Romboutsia* and fungi, *Alternaria, Aureobasidium, Cladosporium, Epicoccum, Mycospherella, Sigarispora,* and *Symmetrospora* were signature above ground taxa. *Burkholderia* and *Streptomyces* were present in both below and aboveground tissue.

The identity of the most prevalent ASVs that were unique to each biocompartment or shared across biocompartments were determined using Venn diagrams with filtering consisting of ≥50% incidence for the belowground compartments and >10% of bacterial flush and flower samples, with a relative abundance >0.1%. This filtering narrowed the dataset to a total of 541 ASVs (370 bacterial and 171 fungal ASVs). The rhizosphere was the biocompartment that displayed the highest number of unique filtered ASVs for both bacteria and fungi (258 ASVs total = 48%), whereas root, flower, and flush only showed 4.4%, 1.1%, and 0.6% of unique ASVs for the combined fungi and bacteria datasets,

respectively. The fungal *Epicoccum* and bacterial *Acinetobacter Aquabacterium, Gilliamella, Kocuria, Romboutsia, Snodgrassella,* and *Tychonema* ASVs were biomarkers of the above-ground tissues as they were only found in the flush, flower or both. The fungal *Beauveria, Fusarium*/*Giberella, Fusicola, Mortierella, Setophaeosphaeria, Solicoccozyma,* and bacterial *Bradyrhizobium, Cupriavidus, Mucilagnibacter, Pseudathrobacter, Steroidobacter* ASVs were biomarkers of the belowground citrus as they were only found in the root, rhizosphere, or both. The majority (79%) of total number of fungal and bacterial ASVs inhabiting the roots (112 ASVs) were of soil/rhizosphere origin. Only 4.8% of the total number bacterial and fungal ASVs (26 ASVs total) were capable of colonizing at least one of the below and above-ground compartments highlighting their ubiquitous nature and included ASVs belonging to the fungal genera *Alternaria, Cladosporium, Mycosphaerella, Neocosmospora, Sigarispora*, and *Symmetrospora*, and to the bacterial genera *Actinoplanes, Bacillus, Burkholderia, Firmicutes, Mesorhizobium, Pseudomonas, Massilia, Sphingomonas,* and *Streptomyces*.

2.4 Discussion

The aim of this study was to profile the citrus microbiome at the floral bud development stage, to capture the microbial community composition of the flower and the flush, and to better comprehend the root microbial assembly process. We hypothesize that citrus trees host beneficial microbes that could be utilized to support tree health and productivity. We focused on a single orchard in California, and spatially profiled for two

consecutive years the citrus microbiome across a continuum of five biocompartments from the host ectospshere to its endosphere at one specific phenological phase. The floral bud development is a pivotal phenophase because of its implication in the vegetative and reproductive cycles of the tree. During this phenophase, trees undergo drastic physiological shifts with respect to carbon reallocation, water dynamics, and phytohormones production (Goldschmidt and Koch, 2017; Agustí et al., 2022), and those changes impact microbial communities. Studies have proposed that the microbiome of plant organs is composed of core taxa across the tree phenophases that may serve a community-stabilizing function and transient phenophase-specific taxa that have more specialized functions in the community (Ginnan et al., 2022). Thus, we are more likely to identify, during the floral bud development, taxa with important biological functions that could have a broad impact on tree health and productivity. Furthermore, flush is a tender tissue fed on by several insect pests (http://ipm.ucanr.edu/PMG/GARDEN/FRUIT/citrus.html), including the asian citrus psyllid, the vector of *C*Las, the causal bacterial agent of Huanglongbing. Mining the microbiome of the shoot flush may help with the identification of biocontrol agents that could be deployed against citrus pests and diseases.

The microbial biodiversity of citrus trees was majorly located in the plant rhizosphere, with the bacteriome showing higher taxonomic richness than the mycobiome (Blaustein et al., 2017; Ginnan et al., 2020). Trees were predominantly colonized across all compartments by Ascomycota fungi and Proteobacteria (Bai et al. 2019; Trivedi et al. 2010; Passera et al. 2018; Xu et al., 2018) but microbial composition within those groups

was vastly different between the above and below ground compartments as indicated by B-diversity plots. Microbial diversity in the flush and flower was low and Ascomycota and Proteobacteria represented overwhelmingly 80% of the microbial relative abundance. In contrast, microbial assemblage belowground was more complex, especially for bacteria, and included a wide range of taxonomic groups spanning several bacterial phyla. Our data indicated that a minority of taxa (5%) were capable of colonizing both below and above-ground habitats. We defined core taxa as genera prevalent in at least 50% of our samples and with a relative abundance of at least 1% and with ASVs within those groups capable of colonizing at least one of the below and above-ground tissue analyzed. Based on these criteria we found that fungi *Alternaria*, *Cladosporium, Fusarium* (syn. *Gibberella*, *Neocosmospora*), *Mycosphaerella, Sigarispora*, and *Symmetrospora*, and the bacterial genera *Actinoplanes*, *Bacillus*, *Burkholderia*, *Firmicutes*, *Massilia*, *Mesorhizobium*, *Pseudomonas*, *Sphingomonas,* and *Streptomyces* were core members of the citrus holobiont. Many of these bacterial taxa are known plant growth promoters and biocontrol agents and can provide fitness advantages to the host (Lemanceau et al., 2017). However, the benefits of the fungal taxa remain elusive. Most taxa within these genera are known pathogens to citrus. *Fusarium solani* can cause wood dry rot (Sandoval-Denis et al., 2018), while *Alternaria alternata* and *A. arborescens, Cladosporium ramotenellum*, and *Mycosphaerella citri* can blemish the fruit. Although, the causal agent of greasy spot, *Mycosphaerella citri,* has not been reported in California. *Sigarispora*, and *Symmetrospora* have been found in several habitats with no known function. Only *Cladosporium cladosporioides* isolated from citrus was shown to inhibit *Liberibacter*

crescens, a culturable surrogate of CLas (Blacutt et al., 2020), and could provide some benefits to the host. Deeper amplicon-based sequencing will help with the naming of the fungal species associated with citrus which may provide some information about their lifestyle. Large-scale sampling coupled with metagenomics, metatranscriptomics, and metabolomics will shed light on the geographical distribution and functional attributes of the core fungal taxa within the citrus holobiont, although this approach still remains limited by the availability of reference genomes (Xu et al., 2018).

The root-associated microbiome of a healthy plant is a relatively stable ecosystem because roots are immersed in a buffered environment (the soil) that is not under the direct constraints of extreme weather conditions and agricultural practices, unlike above-ground plant compartments. Roots are also less affected by the host phenological changes unlike the flower and flush tissues (Ginnan et al., 2022). Here we provide a better understanding of the citrus root endosphere assemblage in citrus and describe the acquisition of specific microbes along the soil-rhizosphere-root continuum that could be targeted for bioinoculants. Root microbial assembly has been described as a two-step process, involving the acquisition of specific microbes from the soil to the rhizosphere and a host-driven sorting step mechanism that subsets specific microbes into the root (Bulgarelli et al., 2013). Our data clearly support this mechanism, with shifts in microbial composition from the bulk soil to the rhizosphere and to the root. The core microbiome of the rhizosphere was composed of the aforementioned core members of the citrus holobiont, plus signature underground taxa that included the bacteria *Bradirhizobium*, *Cupriavidus*, *Mucilaginibacter*, *Rhizobium* and *Steroidobater*, and fungi *Fusicola*,

Glomus, and *Rhizophagus*. Comparative profiling of bulk soil and rhizosphere samples collected across distinct biogeographical regions from six continents also supported that these taxa were enriched in the rhizosphere (Xu et al., 2018). Root exudates act as signal molecules and food sources for the selective recruitment of microbes from bulk soil in exchange for increased nutrient assimilation and improved tolerance against abiotic and biotic stresses. Metagenomic sequencing of soil and rhizosphere communities clearly showed that the functional traits enriched in the rhizosphere influenced microbial assembly and plant health (Xu et al., 2018). Specifically, enriched functional attributes affecting microbial assembly were involved in plant-microbe and microbe-microbe interactions (e.g., antimicrobial synthesis, biofilm formation), nutrient acquisition of microbes, and bioremediation of aromatic compounds. Enriched functional traits that benefit the host were involved in nutrient acquisition, hormonal balance, and pathogen inhibition.

The microbial communities inhabiting the citrus root endosphere mostly originated from the rhizosphere (79% of ASVs), However, we measured a threefold and fivefold decrease in richness between the rhizosphere and the root endosphere for both fungi and bacteria, respectively, which support previous findings (Reinhold-Hurek et al., 2015; Wang et al., 2020). The selective forces imposed by the plant host in the endorhiza are a bottleneck to biodiversity as observed in several plant systems (Fitzpatrick et al., 2018a; Deyett and Rolshausen, 2020; Zhang et al., 2021). Interestingly, the backbone of the root endospheric communities was made of taxa from the core rhizosphere microbiome, suggesting that similar functional traits should overlap between the rhizosphere and root

endosphere. We measured a strong enrichment pattern for some taxa including the bacteria *Actinoplanes, Burkholderia, Mucilaginibacter, Rhizobium, Rhodobacter, Glomus, Rhizophagus,* and *Neocosmospora (Fusarium solani* complex). All five bacteria can promote plant growth by way of fixing nitrogen, solubilizing phosphorus, producing phytohormone production, and increasing abiotic stress tolerance as well as and protect against pathogens by producing antimicrobial compounds or priming plant defense (Kawagushi et al, 2012; Zhang et al., 2017; Orsi et al., 2021; Boukhatem et al., 2022; Fan and Smith, 2022). *Glomus* and *Rhizophagus* are arbuscular mycorrhizal fungi (AMF) that form symbiotic associations with the plant host facilitating water and nutrient acquisition (phosphorus and nitrogen) and increasing the host defense against pathogen attack (Hohmann and Messmer, 2017; Chen et al., 2018). AMF has been commonly found in association with citrus in US orchards (Xi et al., 2022). The genus *Neocosmospora* (*Fusarium solani* species complex) contains saprobes, endophytes, and pathogens and can cause root rot in citrus (Sandoval-Denis et al., 2018, 2019) and is the only organism that is not enriched in the root by a host-driven selection mechanism. The root endosphere microbial communities were also composed of a minority of above ground that may also provide additional functional benefits to the host.

In contrast to rhizocompartments, above-ground microorganisms associated with plants are directly exposed to adverse environmental conditions (rainfall, heat, and UV radiation) and agricultural practices (agrochemical sprays) and are influenced by the host phenology. Those factors impose strong selective pressure on microbiome diversity and composition (Vorholt, 2012; Ginnan et al., 2022). The citrus flower and flush displayed

low microbial richness and communities were composed of the ubiquitous bacteria part of the core citrus holobiont and signature above-ground organisms. Ecologically important genera within the flush and flower microbiome included the bacteria *Acinetobacter* and *Sphingomonas* which were already predicted to be keystone taxa of citrus leaves (Ginnan et al., 2022). *Acinetobacter* has been reported to be highly abundant in the floral nectar microbiome of *Citrus paradisi* and other plant species (Fridman et al., 2012; Alvarez-Perez and Herrera, 2013). *Sphingomonas* is occurring widely in the citrus rhizosphere (Xu et al, 2018), and was categorized as a core member of the citrus microbiome. Both bacteria were identified in the grapevine sap (Deyett and Rolshausen, 2020). Our data also showed that ASVs for two of the core member taxa, *Burkholderia* and *Streptomyces*, were present in the rhizosphere and colonized all the citrus biocompartments. Together, this supports that microbes can move acropetally and basipetal through the xylem and phloem and that above and below ground compartments are connected by way of the plant sap (Compant et al., 2010; Deyett and Rolshausen, 2019, 2020). These bacteria have well-known plant growth-promoting capabilities through phytohormone production, phosphate solubilization, and degradation of organometallic and are also antagonistic towards fungi (Liu et al., 2007; Kang et al., 2009, 2012; Asaf et al., 2020). They have been shown to peak at the flowering stage in citrus and grapevine (Deyett and Rolshausen, 2019; Ginnan et al., 2022). It is tempting to speculate that similar to microbial recruitment mechanisms occurring in the rhizosphere, plants select within its canopy environment bacteria that provide exogenous service to promote reproductive and vegetative cycles in sync with the host phenology. Additional

flush and flower taxa shared with leaf tissues including *Snodgrassella* and *Gilliamella* have been categorized as immigrant taxa introduced by pollinators during dispersal events (Ginnan et al., 2022) because abundant in bee's gut (Powell et al., 2014). Bacteria can be introduced to plants by bees and potentially migrate from the flower to the vascular bundles resulting in systemic movement within the plant (Cellini et al., 2019; Kim et al., 2019) as reported here for soil-introduced bacteria. Interestingly, the fungus *Epicoccum* was the only signature fungus of the above-ground tissue that has been reported as inhibitory to *L. crescens* (Blacutt et al., 2020) and given its ability to colonize the flush, could be explored as a biocontrol to *C*Las for Huanglongbing management.

This study provides significant information about the assemblage of microbial communities in citrus and what constitutes a healthy microbiome. Our results support that the citrus microbiome is composed of core taxonomic groups that appear to be mainly of soil origin and that can systemically colonize trees. In addition, our data indicate microbial niche compartmentalization with specialized taxa capable of colonizing either the above or the below-ground biocompartments but also transient taxa that are in sync with the host phenology and only present during flowering and tree flushing. We identified key plant growth-promoting bacteria (e.g., *Burkholderia, Sphingomonas,* and *Streptomyces*) actively recruited by the host and enriched in all biocomparments that could be harnessed for bioproduct commercialization to improve tree health. We also identify tissue-specific microbes (e.g., *Acinetobacter, Epicoccum*) that could colonize the citrus flush and flower and could enhance tree productivity or management against pests and diseases and notably Huanglongbing. Broad biogeographical sampling and shotgun

metagenomic approach has greatly helped comprehend the structural and functional composition of the citrus rhizosphere microbiome (Xu et al., 2018). The next frontier is to expand this approach to the host endosphere that is more likely to host microbes with bioactive functions and understand how key some of the key beneficial microbes identified here respond to targeted cultural practices. This will help develop recommendations for the industry to improve agricultural practices such as fertilization and pest and disease management. It will also help agrochemical companies select bioinoculants that could be marketed to new technology with improved efficiency.

2.5 Reference

Agustí, M., Mesejo, C., Muñoz-Fambuena, N., Vera-Sirera, F., de Lucas, M., Martínez-Fuentes, A., et al. (2020). Fruit-dependent epigenetic regulation of flowering in Citrus. *New Phytol.* 225, 376–384.

Agustí, M., Reig, C., Martínez-Fuentes, A., and Mesejo, C. (2022). Advances in Citrus Flowering: A Review. *Front. Plant Sci.* 13.

Alvarez-Perez, S., and Herrera, C. M. (2013). Composition, richness and nonrandom assembly of culturable bacterial–microfungal communities in floral nectar of Mediterranean plants. *FEMS Microbiol. Ecol.* 83, 685–699.

Asaf, S., Numan, M., Khan, A. L., and Al-Harrasi, A. (2020). Sphingomonas: from diversity and genomics to functional role in environmental remediation and plant growth. *Crit. Rev. Biotechnol.* 40, 138–152. doi: 10.1080/07388551.2019.1709793.

Bai, Y., Wang, J., Jin, L., Zhan, Z., Guan, L., Zheng, G., et al. (2019). Deciphering bacterial community variation during soil and leaf treatments with biologicals and biofertilizers to control huanglongbing in citrus trees. *J. Phytopathol.* 167, 686–694. doi: 10.1111/jph.12860.

Blaustein, R. A., Lorca, G. L., Meyer, J. L., Gonzalez, C. F., and Teplitski, M. (2017). Defining the core citrus leaf- and root-associated microbiota: Factors associated with community structure and implications for managing huanglongbing (citrus greening) disease. *Appl. Environ. Microbiol.* 83. doi: 10.1128/AEM.00210-17.

Bodenhausen, N., Horton, M. W., and Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. *PLoS One* 8, e56329.

Boukhatem, Z. F., Merabet, C., and Tsaki, H. (2022). Plant growth promoting actinobacteria, the most promising candidates as bioinoculants? *Front. Agron.*, 14.

Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *J. plant Pathol.*, 7–37.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.

Cellini, A., Giacomuzzi, V., Donati, I., Farneti, B., Rodriguez-Estrada, M. T., Savioli, S., et al. (2019). Pathogen-induced changes in floral scent may increase honeybee-mediated dispersal of Erwinia amylovora. *ISME J.* 13, 847–859.

Chen, M., Arato, M., Borghi, L., Nouri, E., and Reinhardt, D. (2018). Beneficial services of arbuscular mycorrhizal fungi–from ecology to application. *Front. Plant Sci.* 9, 1270.

Chiyaka, C., Singer, B. H., Halbert, S. E., Morris Jr, J. G., and van Bruggen, A. H. C. (2012). Modeling huanglongbing transmission within a citrus tree. *Proc. Natl. Acad. Sci.* 109, 12213–12218.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42, 669–678.

Council, N. R. (2010). *Strategic planning for the Florida citrus industry: addressing citrus greening disease*. National Academies Press.

Deyett, E., and Rolshausen, P. E. (2019). Temporal dynamics of the sap microbiome of grapevine under high Pierce's disease pressure. *Front. Plant Sci.* 10, 1246.

Deyett, E., and Rolshausen, P. E. (2020). Endophytic microbial assemblage in grapevine. *FEMS Microbiol. Ecol.* 96. doi: 10.1093/femsec/fiaa053.

Fan, D., and Smith, D. L. (2022). Mucilaginibacter sp. K Improves Growth and Induces Salt Tolerance in Nonhost Plants via Multilevel Mechanisms. *Front. Plant Sci.* 13.

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., and Johnson, M. T. J. (2018a). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proc. Natl. Acad. Sci.* 115, E1157–E1165.

Fitzpatrick, C. R., Lu-Irving, P., Copeland, J., Guttman, D. S., Wang, P. W., Baltrus, D. A., et al. (2018b). Chloroplast sequence variation and the efficacy of peptide nucleic acids for blocking host amplification in plant microbiome studies. *Microbiome* 6, 1–10.

Fridman, S., Izhaki, I., Gerchman, Y., and Halpern, M. (2012). Bacterial communities in floral nectar. *Environ. Microbiol. Rep.* 4, 97–104.

Ginnan, N. A., Dang, T., Bodaghi, S., Ruegger, P. M., McCollum, G., England, G., et al. (2020). Disease-induced microbial shifts in citrus indicate microbiome-derived responses to huanglongbing across the disease severity spectrum. *Phytobiomes J.* 4, 375–387.

Ginnan, N. A., De Anda, N. I., Campos Freitas Vieira, F., Rolshausen, P. E., and Roper, M. C. (2022). Microbial Turnover and Dispersal Events Occur in Synchrony with Plant Phenology in the Perennial Evergreen Tree Crop Citrus sinensis. *MBio*, e00343-22.

Goicoechea, N., Antolin, M. C., and Sánchez-Díaz, M. (1997). Influence of arbuscular mycorrhizae and Rhizobium on nutrient content and water relations in drought stressed alfalfa. *Plant Soil* 192, 261–268.

Goldschmidt, E. E., and Koch, K. E. (2017). "Citrus," in *Photoassimilate distribution in plants and crops* (Routledge), 797–824.

Gopal, M., Gupta, A., and Thomas, G. V (2013). Bespoke microbiome therapy to manage plant diseases. *Front. Microbiol.* 4, 355.

Gottel, N. R., Castro, H. F., Kerley, M., Yang, Z., Pelletier, D. A., Podar, M., et al. (2011). Distinct microbial communities within the endosphere and rhizosphere of Populus deltoides roots across contrasting soil types. *Appl. Environ. Microbiol.* 77, 5934–5944.

Hohmann, P., and Messmer, M. M. (2017). Breeding for mycorrhizal symbiosis: focus on disease resistance. *Euphytica* 213, 113.

Kang, S.-M., Joo, G.-J., Hamayun, M., Na, C.-I., Shin, D.-H., Kim, H. Y., et al. (2009). Gibberellin production and phosphate solubilization by newly isolated strain of Acinetobacter calcoaceticus and its effect on plant growth. *Biotechnol. Lett.* 31, 277–281.

Kang, S.-M., Khan, A. L., Hamayun, M., Shinwari, Z. K., Kim, Y.-H., Joo, G.-J., et al. (2012). Acinetobacter calcoaceticus ameliorated plant growth and influenced gibberellins and functional biochemicals. *Pak. J. Bot* 44, 365–372.

Kawaguchi, A., Kondo, K., and Inoue, K. (2012). Biological control of apple crown gall by nonpathogenic Rhizobium vitis strain VAR03-1. *J. Gen. plant Pathol.* 78, 287–293.

Kim, D.-R., Cho, G., Jeon, C.-W., Weller, D. M., Thomashow, L. S., Paulitz, T. C., et al. (2019). A mutualistic interaction between Streptomyces bacteria, strawberry plants and pollinating bees. *Nat. Commun.* 10, 1–10.

Lau, J. A., and Lennon, J. T. (2011). Evolutionary ecology of plant–microbe interactions: soil microbial structure alters selection on plant traits. *New Phytol.* 192, 215–224.

Lemanceau, P., Blouin, M., Muller, D., and Moënne-Loccoz, Y. (2017). Let the core microbiota be functional. *Trends Plant Sci.* 22, 583–595.

Li, H., Song, F., Wu, X., Deng, C., Xu, Q., Peng, S., et al. (2021). Microbiome and Metagenome Analysis Reveals Huanglongbing Affects the Abundance of Citrus Rhizosphere Bacteria Associated with Resistance and Energy Metabolism. *Horticulturae* 7, 151.

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

Liu, C. H., Chen, X., Liu, T. T., Lian, B., Gu, Y., Caer, V., et al. (2007). Study of the antifungal activity of Acinetobacter baumannii LCH001 in vitro and identification of its antifungal components. *Appl. Microbiol. Biotechnol.* 76, 459–466.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature* 488, 86–90.

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8, e61217.

Moss, G. I. (1969). Influence of temperature and photoperiod on flower induction and inflorescence development in sweet orange (Citrus sinensis L. Osbeck). *J. Hortic. Sci.* 44, 311–320.

Moss, G. I. (1976). Temperature effects on flower initiation in sweet orange (Citrus sinensis). *Aust. J. Agric. Res.* 27, 399–407.

Olesen, T., Smith, G., and Muldoon, S. J. (2013). Flush development in Tahitian lime. *Aust. J. Bot.* 61, 358–364.

Orsi, E., Beekwilder, J., Eggink, G., Kengen, S. W. M., and Weusthuis, R. A. (2021). The transition of Rhodobacter sphaeroides into a microbial cell factory. *Biotechnol. Bioeng.* 118, 531–541.

Passera, A., Alizadeh, H., Azadvar, M., Quaglino, F., Alizadeh, A., Casati, P., et al. (2018). Studies of microbiota dynamics reveals association of “Candidatus Liberibacter asiaticus” infection with citrus (Citrus sinensis) decline in south of Iran. *Int. J. Mol. Sci.* 19, 1817.

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci.* 110, 6548–6553.

Powell, J. E., Martinson, V. G., Urban-Mead, K., and Moran, N. A. (2014). Routes of acquisition of the gut microbiota of the honey bee Apis mellifera. *Appl. Environ. Microbiol.* 80, 7378–7387.

Rastogi, G., Sbodio, A., Tech, J. J., Suslow, T. V, Coaker, G. L., and Leveau, J. H. J. (2012). Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J.* 6, 1812–1822.

Redford, A. J., and Fierer, N. (2009). Bacterial succession on the leaf surface: a novel system for studying successional dynamics. *Microb. Ecol.* 58, 189–198.

Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., and Hurek, T. (2015). Roots

shaping their microbiome: global hotspots for microbial activity. *Annu Rev Phytopathol* 53, 403–424.

Sagaram, U. S., DeAngelis, K. M., Trivedi, P., Andersen, G. L., Lu, S.-E., and Wang, N. (2009). Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using PhyloChip arrays and 16S rRNA gene clone library sequencing. *Appl. Environ. Microbiol.* 75, 1566–1574.

Sandoval-Denis, M., Guarnaccia, V., Polizzi, G., and Crous, P. W. (2018). Symptomatic Citrus trees reveal a new pathogenic lineage in Fusarium and two new Neocosmospora species. *Persoonia-Molecular Phylogeny Evol. Fungi* 40, 1–25.

Sandoval-Denis, M., Lombard, L., and Crous, P. (2019). Back to the roots: a reappraisal of Neocosmospora. *Persoonia-Molecular Phylogeny Evol. Fungi* 43, 90–185.

Schlaeppi, K., and Bulgarelli, D. (2015). The plant microbiome at work. *Mol. Plant-Microbe Interact.* 28, 212–217. doi: 10.1094/MPMI-10-14-0334-FI.

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12.

STANDER, J. O. P. J. (2015). The Reproductive Phenology of Citrus III: Morphogenesis from Flower to Fruit.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature* 418, 671–677.

Trivedi, P., Duan, Y., and Wang, N. (2010). Huanglongbing, a systemic disease, restructures the bacterial community associated with citrus roots. *Appl. Environ. Microbiol.* 76, 3427–3436.

Trivedi, P., Spann, T., and Wang, N. (2011). Isolation and characterization of beneficial bacteria associated with citrus roots in Florida. *Microb. Ecol.* 62, 324–336.

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10, 828–840. doi: 10.1038/nrmicro2910.

Wang, X., Wang, M., Xie, X., Guo, S., Zhou, Y., Zhang, X., et al. (2020). An amplification-selection model for quantified rhizosphere microbiota assembly. *Sci Bull* 65, 1436–1439.

Weinert, N., Piceno, Y., Ding, G.-C., Meincke, R., Heuer, H., Berg, G., et al. (2011). PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many common and few cultivar-dependent taxa. *FEMS Microbiol. Ecol.* 75, 497–506.

Wu, Y., Qu, M., Pu, X., Lin, J., and Shu, B. (2020). Distinct microbial communities among different tissues of citrus tree Citrus reticulata cv. Chachiensis. *Sci. Rep.* 10. doi: 10.1038/s41598-020-62991-z.

Xi, M., Deyett, E., Ginnan, N., Ashworth, V. E. T. M., Dang, T., Bodaghi, S., et al. (2022). Arbuscular mycorrhizal fungal composition across US citrus orchards, management strategies, and disease severity spectrum. *bioRxiv*.

Xu, J., Zhang, Y., Zhang, P., Trivedi, P., Riera, N., Wang, Y., et al. (2018). The structure and function of the global citrus rhizosphere microbiome. *Nat. Commun.* 9. doi: 10.1038/s41467-018-07343-2.

Zhang, Y., Trivedi, P., Xu, J., Caroline Roper, M., and Wang, N. (2021). The Citrus Microbiome: From Structure and Function to Microbiome Engineering and beyond. *Phytobiomes J.* 5, 249–262. doi: 10.1094/PBIOMES-11-20-0084-RVW.

Zhang, Y., Xu, J., Riera, N., Jin, T., Li, J., and Wang, N. (2017). Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. *Microbiome* 5. doi: 10.1186/s40168-017-0304-4.

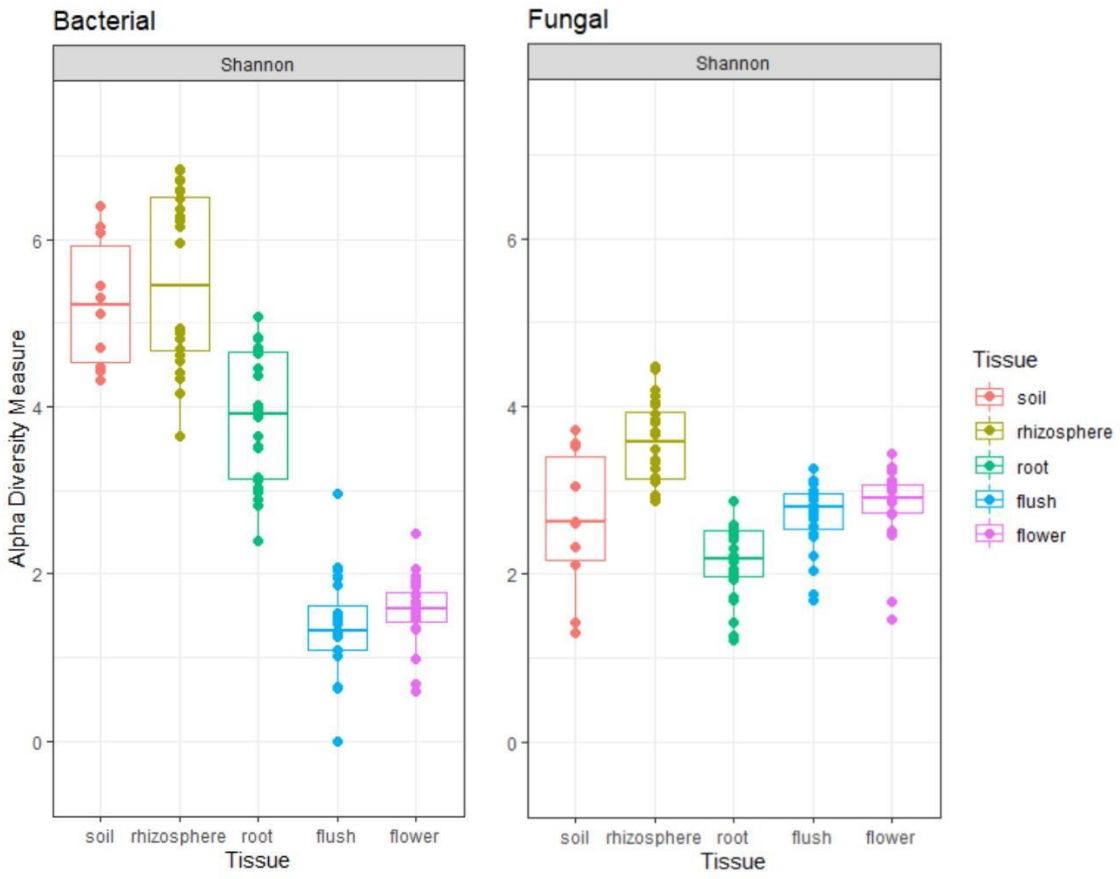

Figure 2.1 Shannon alpha-diversity plots for bacteria and fungi within five different citrus biocompartments (soil, rhizosphere, root, flush, and flower).

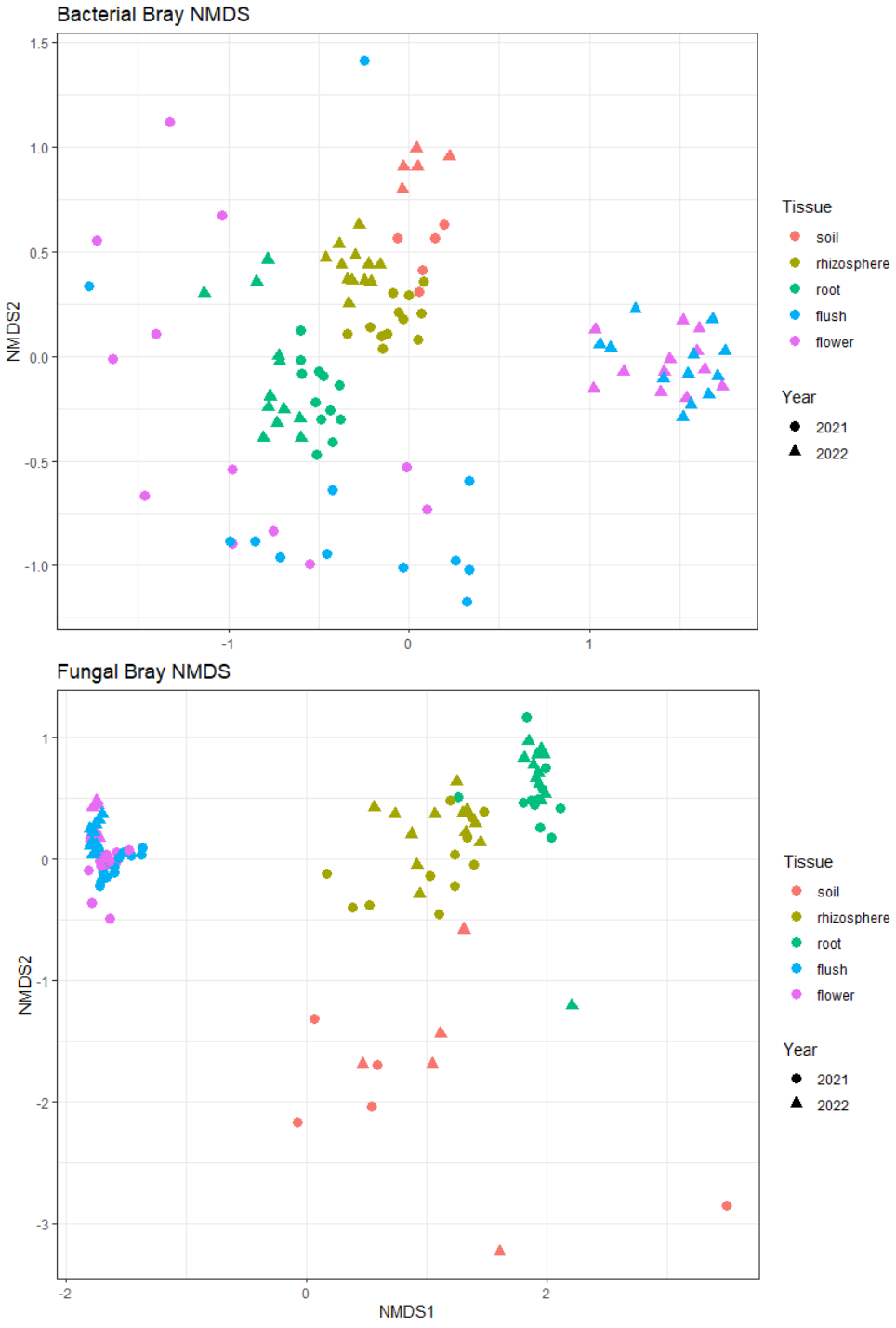

Figure 2.2 Bray–Curtis beta diversity for bacteria and fungi. Points represent individual sample communities for one biocompartment from one citrus tree at one year. Points are colored by biocompartment and shaped by the year collected.

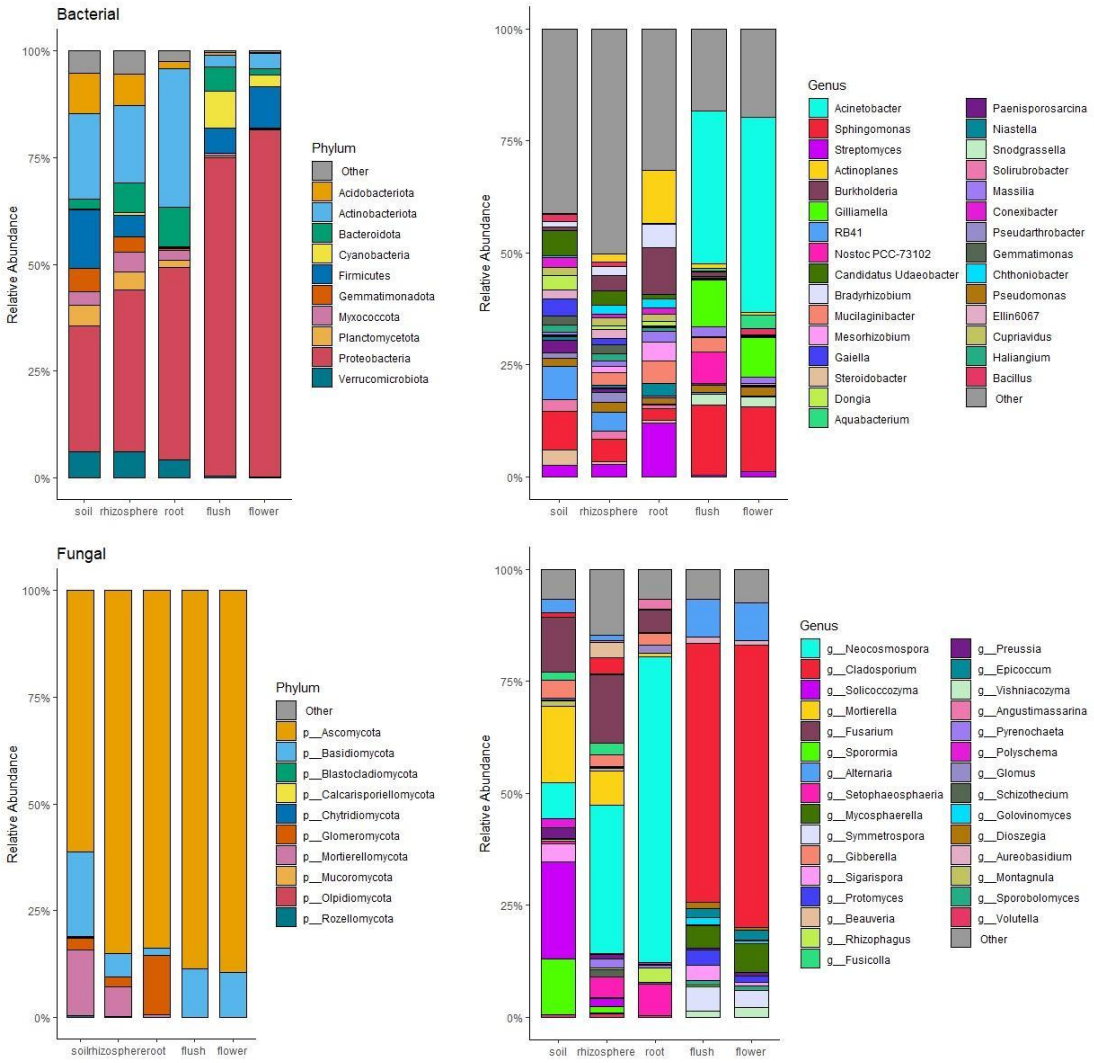

Figure 2.3 Relative abundant bar chat of bacteria and fungi community at phylum and genus level within individual citrus biocompartment (soil, rhizosphere, root, flush, and flower). Only the top 10 phyla and top 30 genera occurring at ≥1% relative abundance are displayed.

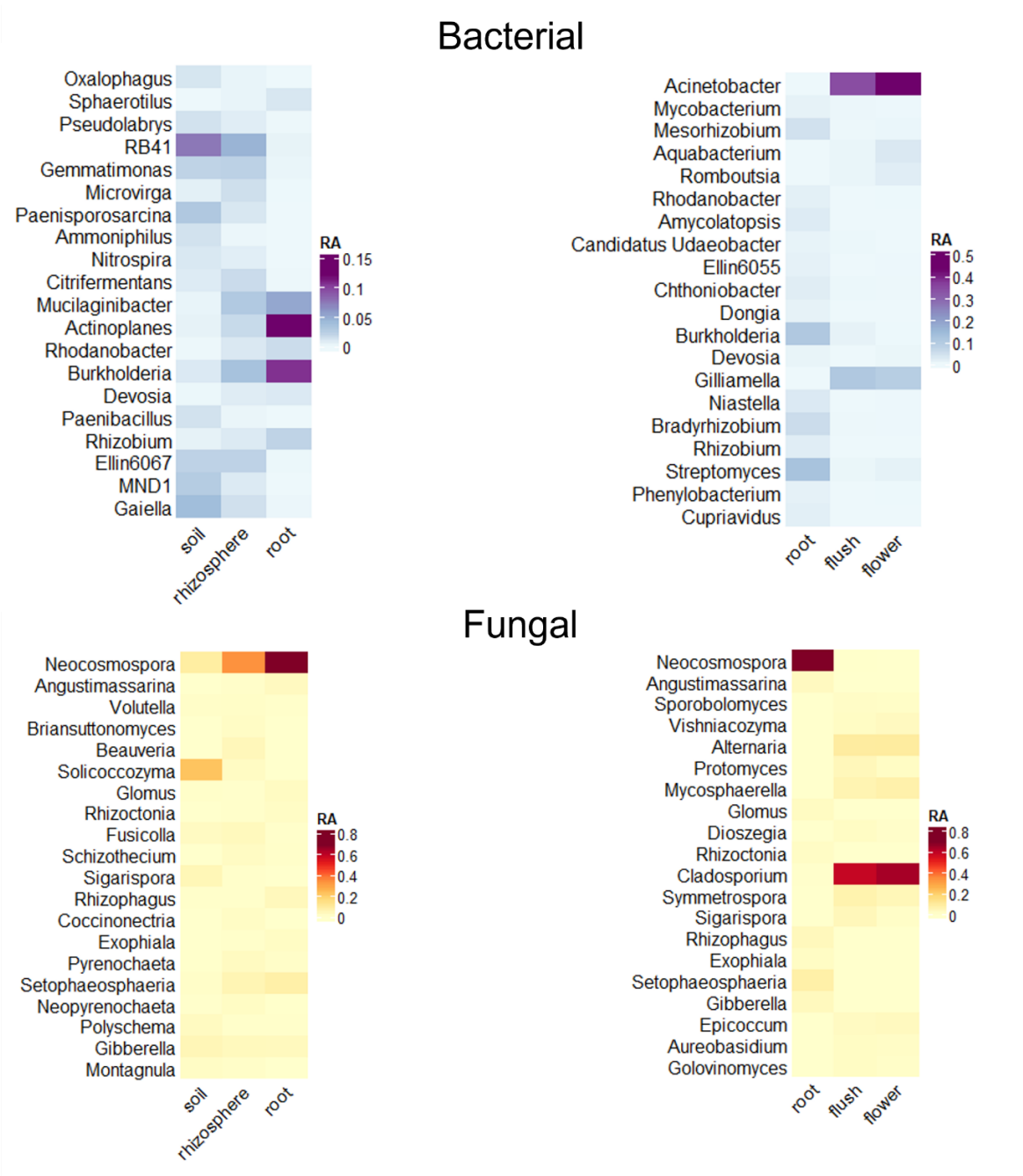

Figure 2.4 Relative abundance heat maps of significant taxa as determined through DESeq2 analyses. Data grouped by belowground-specific (root, rhizosphere, soil) and below to above ground (root, flush, flower) habitats in Bacteriome and mycobiome. Data were filtered to a $P < 0.01$ cutoff and log2 fold change of >5 or <−5. Only genera occurring at ≥ 1% with the top 20 highest log2 fold changes are displayed.

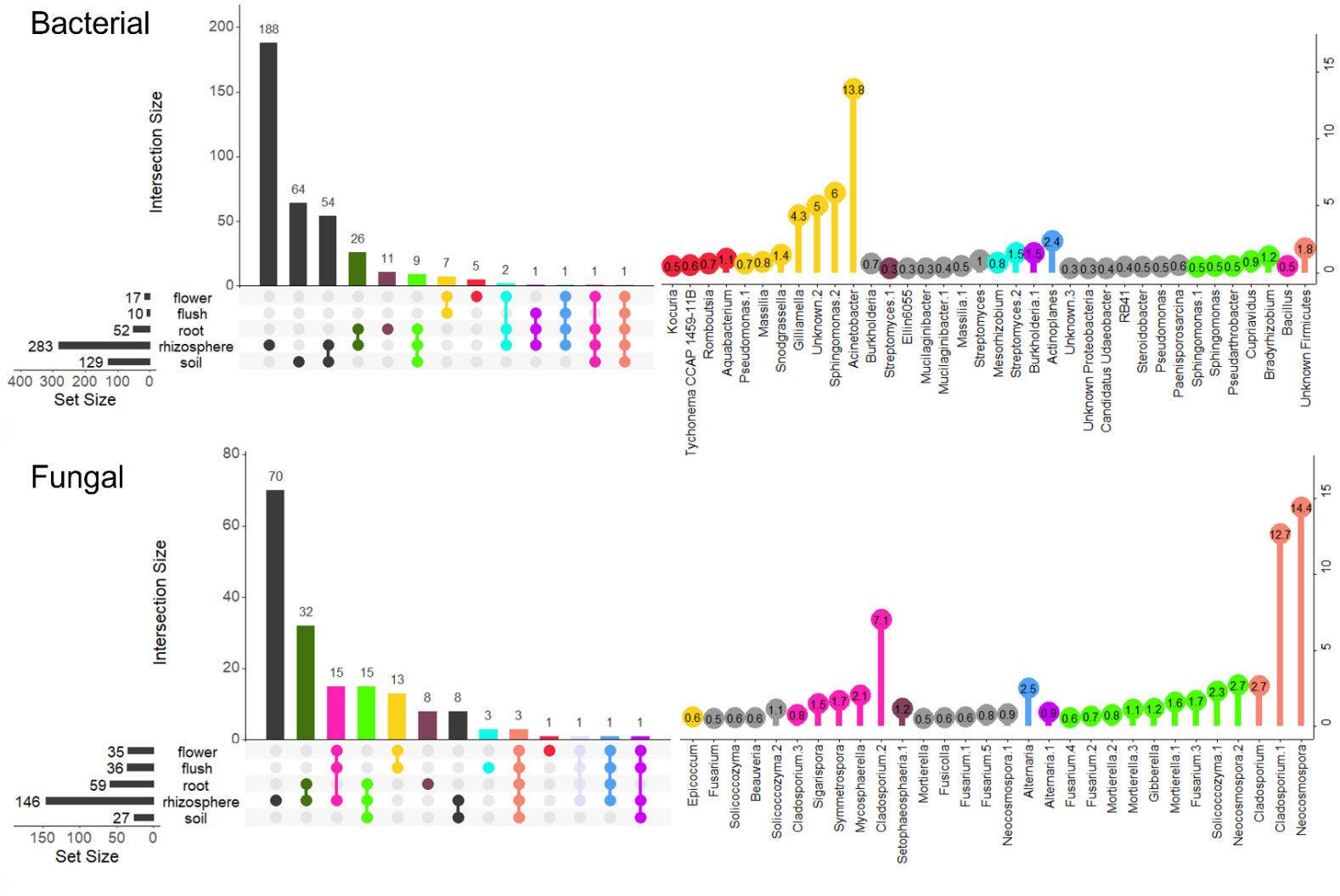
Bacterial
Intersection Size
188
64
54
26
11
9
7
5
2
1
1
1
1
17
10
52
283
129
flower
flush
root
rhizosphere
soil
Set Size
Kocuria
Tychonema CCAP 1459-11B
Romboutsia
Aquabacterium
Pseudomonas.1
Massilia
Snodgrassella
Gillilamella
Unknown.2
Sphingomonas.2
Acinetobacter
Burkholderia
Streptomyces.1
Ellin6055
Mucilaginibacter
Mucilaginibacter.1
Massilia.1
Streptomyces
Mesorhizobium
Streptomyces.2
Burkholderia.1
Actinoplanes
Unknown.3
Unknown Proteobacteria
Candidatus Udaeobacter
RB41
Steroidobacter
Pseudomonas
Paenisporosarcina
Sphingomonas.1
Sphingomonas
Pseudarthrobacter
Cupriavidus
Bradyrhizobium
Bacillus
Unknown Firmicutes
Fungal
Intersection Size
70
32
15
15
13
8
8
3
3
1
1
1
1
35
36
59
146
27
flower
flush
root
rhizosphere
soil
Set Size
Epicoccum
Fusarium
Solicoccozyma
Beauveria
Solicoccozyma.2
Cladosporium.3
Sigarispora
Symmetrospora
Mycosphaerella
Cladosporium.2
Setophaeosphaeria.1
Mortierella
Fusicolla
Fusarium.1
Fusarium.5
Neocosmospora.1
Alternaria
Alternaria.1
Fusarium.4
Fusarium.2
Mortierella.2
Mortierella.3
Gibberella
Mortierella.1
Fusarium.3
Solicoccozyma.1
Neocosmospora.2
Cladosporium
Cladosporium.1
Neocosmospora

Figure 2.5 Prevalence Venn diagrams at ASV level showing overlapping taxa: genera that occur in ≥50% of all samples (>10% of bacterial flush and flower samples) from each biocompartment. Intersections of (A) bacterial and (C) fungal genera and associated with all biocompartment combinations. (B) (D) Relative abundant for the genera colored by intersections in (A) (C). Only genera occurring at ≥ 0.5% relative abundance are displayed.

3 Geographic Location, Management Strategy and Huanglongbing Disease Affect Arbuscular Mycorrhizal Fungal Communities Across US Citrus Orchards.

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are biotrophic organisms forming symbiotic associations with more than 70% of land plants across a broad range of terrestrial ecosystems (Smith and Read 2008; Brundrett and Tedersoo 2018). The nature of the symbiosis spans from mutualistic to parasitic (Johnson et al. 1997). The mutualistic symbiosis resides in a trade-off between a more efficient root acquisition of water and nutrients (especially phosphorus) via the mycorrhizal hyphal network, in exchange for photo-assimilated carbon. The outcome of this interaction often results in improved plant environmental fitness with increased tolerance to biotic and abiotic stresses (Hohmann and Messmer 2017; Chen et al. 2018). In addition, AMF improve soil structure by forming stable soil aggregates thereby limiting erosion and leaching of nutrients (Wilson et al. 2009; Chen et al. 2018). The parasitic interaction between plants and mycorrhizal fungi is only considered when net cost of the symbiosis exceeds net benefits (Johnson et al. 1997).

Plant parts above and below ground are intricately connected and the health status of the root system often determines plant growth and productivity. The rhizosphere microbial diversity is a biomarker of soil fertility and plays a central role in sustainable agricultural systems (Mäder et al. 2002; Hartmann et al. 2015). Low input agriculture

systems (organic and biodynamic farming) rely on soil biological metabolism and function to support soil fertility and plant root health. In contrast, intensive farming practices characterized by monoculture, input of synthetic agrochemicals, and/or soil disturbance generally leads to degradation of soil ecosystem and erosion of AMF biodiversity (Verbruggen et al., 2010). AMF communities are also affected by soil types and land use intensity and AMF community composition is often characterized by a collection of 'specialist' taxa capable of colonizing specific soil habitats and 'generalist' taxa associated with a wide range of diverse ecosystems (Oehl et al., 2003). (Oehl et al. 2010; Verbruggen et al. 2010). Understanding the factors that shape AMF community assemblage under agricultural constraints could lead to deployment of improved sustainable practices.

Citrus is a high-value crop and one of the most popular and widely grown fruit trees globally. It is praised for its nutritional value and benefits to human health as a source of vitamins, fibers, and minerals. Citrus accounts for 16% of the total value of U.S. fruit production (Li et al. 2020) with California and Florida leading the nation's fresh fruit and juice markets, respectively. Symbiotic associations with AMF have been reported in all citriculture production areas and AMF communities are shaped by edaphic characteristics, orchard management practices, and host variety and age (Nemec et al. 1981; Franca et al. 2007; Wang et al. 2012; Song et al. 2015). Adopting low input farming practices for citrus at a large geographic scale has been challenging because of Huanglongbing (HLB), a disease associated with an invasive phloem-limited bacteria in the *Candidatus* Liberibacter genus (i.e., *C*. L. *asiaticus*, *americanus*, and *africanus*)

(Bové 2006). Symptoms of HLB include asymmetrical blotchy mottling of leaves, yellow shoots, thinning of the canopy, wood dieback, with fruits appearing small, off colored, and lopsided with a bitter taste (Bové, 2006). In the U.S., *C.* L. *asiaticus* (*C*Las) is the primary causal agent of citrus HLB and was first detected in Florida in 2005 and California in 2012. In California, the disease has only been reported in residential areas in the southern part of the state. In contrast, the disease has now become endemic to Florida, and the citrus industry has already suffered a 74% decline in production with losses amounting to over $1 billion annually (Court et al. 2018; Li et al. 2020). The pathogen is vectored by an invasive insect (*Diaphorina citri;* the Asian citrus psyllid) and disease management has been mostly achieved by intensive regimens of synthetic insecticide applications to control insect populations (Boina and Bloomquist 2015). In heavily affected orchards, trees are also treated with antibiotics (oxytetracycline and streptomycin) to reduce levels of pathogen inoculum reservoirs (Hu et al. 2018). Those practices have raised environmental concerns due to the risk of unintended consequences for biodiversity and selection for resistance in bacterial and insect populations (Wood and Goulson 2017; McKenna 2019).

In HLB-impacted orchards, the tree rhizosphere suffers from microbial dysbiosis and root collapse, thereby weakening the host and its defense response against attack from other pathogens (Fan et al. 2013; Ginnan et al. 2020). Specifically, a study from Florida found that high relative abundance of Glomeromycota correlated with healthier trees (Ginnan et al. 2020), although the ITS2 amplicon used in the study limited the resolution of AMF taxonomy and may have omitted key taxa (Lekberg et al. 2018) or

biased the identification towards specific taxonomic groups within the Glomeromycota (Davison et al. 2015). AMF can provide protection against citrus root diseases (Watanarojanaporn et al. 2011; Tian et al. 2021) and developing practices that support their biodiversity can offer new ground for management strategies. Previous studies have found at least seven genera of AMF associated with citrus roots (*Acaulospora, Entrophospora, Gigaspora, Glomus, Pacispora, Sclerocystis,* and *Scutellospora*), yet little is known about how these communities vary across geographies or management practices. Moreover, many of these studies are morphological-based and may have underrepresented AMF diversity that can be captured with sequencing approaches (Stefani et al. 2020).

Citrus is an emblematic specialty crop to US agriculture. In the wake of the economic and environmental challenges posed by HLB, alternative strategies to farming citrus should be considered. Here we tested how the two distinct climatic zones within the continental US, where citrus is primarily grown (California and Florida), influenced AMF community diversity and composition. Within each zone, with further evaluated how conventional farming and disease affected AMF diversity and composition. Citrus root-associated AMF were assessed by high-throughput sequencing of the SSU rRNA gene (Lee et al. 2008; Dumbrell et al. 2011). Orchards in California, where commercial HLB-positive trees have not been detected, were selected according to the farming practices (organic vs. conventional). Orchards in Florida were chosen according to severity of HLB disease symptoms expression (mild, moderate, severe). This study provides insightful information about AMF dynamics within a major perennial

agroecosystem and identifies putative generalist taxa that could be exploited for agricultural purposes.

3.2 Materials and Methods

Root sample collection: Root samples were collected from 88 trees in ten citrus orchards located in Florida (ten trees per orchard) and California (eight trees per orchard). Roots were collected 0.5 m away from each side of the tree trunk following published protocols (Ginnan et al. 2020). In Florida, 40 root samples were collected in March 2017 from four conventional orchards (Table 1). All trees were rated for HLB symptoms using a disease rating scale ranging from mildly to moderately and severely symptomatic. In California, 48 root samples were collected in October 2017 from four conventional and two organic orchards (Table 1). Gloves were changed and clippers and shovels were sterilized with 30% household bleach between each sampled tree. All samples were immediately placed in bags on ice in a cooler for transit to the laboratory. Root samples were rinsed with autoclaved purified water (Barnstead Mega-Pure System MP-6a, Thermo Fisher Scientific, Waltham, MA, USA) and approximately 5 g of rinsed root tissue was placed into 50 ml conical tubes, stored at -80°C, and then lyophilized (Labconco FreeZone 4.5L, Kansas City, MO) for 16 to 20 hours. Root samples collected in Florida were shipped to UCR under USDA permit #P526P-16-00352 on dry ice.

DNA extraction, library construction and sequencing: DNA was extracted from roots according to published protocols (Ginnan et al. 2020). Frozen and freeze-dried roots were crushed into small pieces (<0.5 cm) with sterile stainless-steel spatulas on dry-ice, and

100 mg of freeze-dried tissue was transferred to 2 ml microcentrifuge tubes (Eppendorf Safe-Lock tubes; Eppendorf, Hamburg, Germany) containing a single 4 mm stainless-steel grinding ball (SPEX SamplePrep, Metuchen, NJ, U.S.A.). Samples were chilled at −80°C for 15 min, then pulverized to a powder using a 2010 Geno/Grinder (SPEX SamplePrep) at 1,680 rpm for 20 to 30 seconds, twice. Then, 1 ml of 4 M guanidine thiocyanate buffer was added to the pulverized root samples. Samples were incubated at 4°C for 15 min and subsequently centrifuged for 1 h at 17,500 × g. DNA was isolated using the MagMAX-96 DNA Multi-Sample Kit (Thermo Fisher Scientific) with the protocol "MagMAX™ Express-96 Magnetic Particle Processor". The final DNA was eluted in 100 ml of DNA elution buffer and stored at −20°C prior to Illumina library construction.

DNA was PCR-amplified as previously described (Phillips et al. 2019) targeting the 18S region using the universal eukaryote WANDA and the Glomeromycotina-specific AML2 primer sets (Lee et al. 2008; Dumbrell et al. 2011). PCR was conducted in a two-step procedure (Berry et al. 2012) in which first-round amplifications were carried out with primers possessing universal tails synthesized 5′ to the locus-specific sequences (Alvarado et al. 2018) and second round-amplifications ligated Illumina MiSeq flowcell adapters and barcodes (Phillips et al. 2019). PCR1 included 1 µl of template DNA, 12.5 µl AccuStart II PCR ToughMix (2X) (Quantabio, Beverly, MA), 0.5 µl of each primer (10 µM), and 10.5 µl nuclease-free water, resulting in a 25 µl reaction. Thermocycler conditions for PCR1 were as follows: 2 min at 94°C followed by 29 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 68°C. Reaction products were verified on a 1% agarose gel

and purified using the AMPure XP magnetic Bead protocol (Beckman Coulter Inc., Brea, CA, USA). PCR2 was performed in a 25 µl reaction, with 1 µl of the undiluted purified PCR1 product, 6.5 µl AccuStart II PCR ToughMix (2X) (Quantabio, Beverly, MA), 2.5 µl of each barcode primer (1 µM), and 12.5 µl nuclease-free water. Thermocycler conditions for PCR2 were as follows: 2 min at 94°C followed by 9 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C. We checked indexed PCR products on an agarose gel and pooled the products by band strength as previously established (Glassman et al. 2018) with 1 µl for strong bands, 2 µl for medium bands, and 3 µl for weak bands prior to AMPure bead purification. The purified library was quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and quality checked with an Agilent BioAnalyzer 2100 for size and concentration and sequenced with Illumina MiSeq NanoV2 (2 x 250 bp) at the UC Riverside Institute for Integrative Genome Biology. However, there was insufficient overlap between the read pairs to assemble the entire SSU ribosomal RNA amplicon, and thus we only used the forward sequence for phylogenetic purposes and taxa assignment. Sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under accession number PRJNA839101.

Bioinformatics and taxonomy assignment: Initial quality filtering of sequences was done using Trimmomatic (Bolger et al. 2014) truncating reads once the average quality of 5 consecutive base pairs dropped below a quality score of 20. Sequence reads were further filtered using DADA2's recommended parameters (maxN= , maxEE= , truncLen=). DADA2 (v 1.14.1), . DADA2 default parameters were also used to dereplicate, learn error rates, and create an amplicon sequence variant (ASV) table (Callahan et al. 2016).

Samples with less than 1,000 reads were removed, as were taxa not identified as fungi via the NCBI database. Rare taxa, defined as taxa which were prevalent in less than 2% of samples were also removed. Taxonomy was assigned using BLASTN (Altschul et al. 1990) and the MaarjAM database (downloaded on April 1, 2020; https://maarjam.ut.ee/; Öpik et al. 2010) using a cut-off e-value of 1e-50 and assigning virtual taxa (VT) based on lowest e-value. MaarjAM is an AMF curated database that is standardized, comparable across research projects, and preserved in time (Lekberg *et al.* 2018). Sequences were aligned in Muscle v.3.7 (Edgar 2004) implemented on the CIPRES Gateway (Miller *et al.* 2010) using default parameters. The alignment included the VT sequences as well as available consensus sequences from Krüger et al. (Krüger *et al*. 2012) and updated by Stefani *et al.* (Stefani *et al.* 2020) and sequences from NCBI GenBank. Genus names were assigned through the curation of a maximum likelihood bootstrap tree constructed in RAxML v8.2.12 (Stamatakis 2014) implemented on the CIPRES Gateway (Miller et al. 2010) using a GTRGAMMA evolutionary model of nucleotide substitution and with branch support inferred using 1000 bootstraps. The tree was rooted using *Paraglomus occultum* as the outgroup as per Krüger *et al.* (Krüger et al. 2012). The consensus tree was visualized and annotated in iTOL (Interactive Tree of Life, https://itol.embl.de/; (Letunic and Bork 2019)).

Statistical analyses and data visualization: We considered one tree as a single replicate for the three datasets, geographical location (Florida= 34 trees; California= 36 trees), cultural practice (organic= 15 trees; conventional= 21 trees) and HLB severity (mild= 12 trees; intermediate= 10 trees; severe= 11 trees). R v4.1.1 (R Core Team, 2021) was used

to perform statistical analysis and data visualization with the aid of the phyloseq v1.36.0 (McMurdie and Holmes 2013) and ggplot2 v3.3.5 packages (Wickham 2016). Alpha diversity was estimated on untransformed data, as the number of observed taxa of each sample. Statistical significance was calculated by a generalized linear model using Poisson regression and statistical significance on pairwise comparison was performed through Tukey's test using the multcomp packages v1.4.17 (Hothorn et al. 2008). Beta-diversity plots were created on proportionally transformed data using the Bray-Curtis dissimilarity matrix and NMDS ordination matrix using the Vegan package v2.5.7 (Oksanen et al. 2020). Permutational analysis of variance (PERMANOVA) as implemented by the vegan package function *adonis* (with 999 permutations) was run to determine statistical differences in community composition between categorical variables (Anderson, 2008). Pairwise perm manova using the RVAideMemoire package v09-81-2 was run for comparison between groups (Hervé 2018) Data was transformed using the variance stabilization method in the DESeq package v1.32.0 (Love et al. 2014) and was used in heat maps to compare abundance between categories. For heatmaps, created using ComplexHeatmap v2.9.4 (Gu et al. 2016), the variance stabilized transformed data were aggregated to the VT level and dataset split based on category. VT occurring in fewer than 3 samples of the dataset were removed for ease of visualization. This resulted in 27 taxa in the heatmap comparing California and Florida samples, 15 taxa in the heatmap comparing management strategy and 17 taxa in the heatmap comparing disease rating. For the Venn diagram, a 10% prevalence filtering was applied to find unique and shared taxa amongst categories. Finally, DESeq2 (V1.32.0; (Love et al. 2014)) using the default

Wald test and local fit was used to identify taxa with differential abundance analysis among variables of interest. For this analysis, taxa were aggregated to the VT level. Virtual taxa that had a p-value < 0.01 are represented in the heat maps with a "*" symbol or by colored block.

3.3 Results

Quality filtering of the sequences resulted in 1,085,960 reads and 131 ASVs. The MaarjAM database assigned the 131 ASVs to 32 unique VT, and with one sequence left unidentified. The 33 representative VT sequences were aligned to 58 consensus sequences from Krüger *et al.* (Krüger et al. 2012) and updated by Stefani *et al.* (Stefani et al. 2020) and 12 sequences from GenBank, resulting in a total of 103 sequences and an alignment length of 756 nucleotides. Within this alignment, the portion corresponding to the shorter (220 bp-long) sequences of the 33 VT was 239 bp in length and consisted of 93 conserved, 136 variable, 103 parsimony-informative, and 32 singleton sites.

The maximum likelihood phylogenetic analysis that included several taxa from Krüger et al. (Krüger et al. 2012) and the same outgroup (*Paraglomus occultum*), yielded a similar tree topology with strong bootstrap support at the family level and indicated that all the VT belong to the Glomeraceae (Fig. 1). Taxonomic identification at the genus level was more challenging for some groups given the short nucleotide sequence length (220 bp) of the VT, but monophyletic clades with good bootstrap support were obtained for *Sclerocystis*, *Glomus*, and *Septoglomus*. The genus *Rhizophagus* formed a weakly supported clade but was part of a well-supported clade with *Sclerocystis.* Similarly,

Funneliformis—though itself not a monophyletic group—formed a strongly supported clade with *Septoglomus.* Support for *Dominikia* was low and good bootstrap support was obtained only for a subclade composed of two VTs (VTX00222 and -125) with *Dominikia indica*, and for a subclade composed of *D. iranica* and VTX00155. Based on the tree phylogeny, we assigned twelve VT (VTX00125, -130, -132, -146, -155, -156, -159, -166, -175, -222, -304 and -unknown) to *Dominikia*, ten VT (VTX00080, -083, -092, -099, -100, -105, -113, -114, -115, -248) to *Rhizophagus*, four VT (VTX00063, -064, -331, -409) to *Septoglomus*, one VT (VTX00197) to *Glomus*, and one VT (VTX00067) to *Funneliformis.* Placement for five VT (VTX00075, -214, -301, -323, -384) remained uncertain as they did not cluster in any of those clades and these were labeled as Glomeraceae species.

We detected AMF in 69 of the total 88 citrus root samples (78%, Table 1). Geographical location did not affect AMF observed richness in California and Florida citrus orchards as indicated by similar alpha diversity indices (15.8 average ASVs in CA vs. 15.7 average ASVs in FL; Fig.2A; $P > 0.05$, Poisson generalized linear model with a pairwise Tukey test), although AMF community composition differed significantly between the two states (Fig. 3A; Adonis $R^2 = 0.176$, $P < 0.001$). Nine virtual taxa were commonly associated with citrus in both Florida and California orchards and represent 'generalists' (Fig. 4) They included two *Dominikia* taxa (VTX00156, -304), three *Rhizophagus* taxa (VTX00092, -100, -248), one *Septoglomus* taxon (VTX00063), and three unknown taxa within the Glomeraceae (VTX00214, -301, -323). The remaining AMF taxa were associated with a single geographical location (VTX00075, -113, -115, -

125, -132, -222, -384 in FL and VTX00064, -067, -105, -130, -155, -175, -331, -409, and -unknown in CA), and under specific management practices or disease phenotype, which could imply specialized functions and/or unique growth needs (Figs. 4-6). Therefore, we refer to these taxa as 'specialists'. Several taxa from both the generalist and specialist groups were differentially abundant across geographical location (Fig. 4; Wald's Test: $P < 0.01$), including three generalist taxa (*Rhizophagus* VTX00092, *Septoglomus* VTX00063, and unknown Glomeraceae VTX00214) and nine specialist taxa, with five from Florida (*Rhizophagus* VTX00113 and -115; *Dominikia* VTX00222 and -125; unknown Glomeraceae VTX00384) and four from California (*Dominikia* VTX00155 and -130; *Septoglomus* VTX00409 and -064).

Orchard management strategy in California significantly affected both AMF richness and composition, with a significant decrease of observed taxa richness in conventional orchards (12.2 average ASVs in conventional vs. 20.7 average ASVs in organic orchards; Fig. 2B; $P < 0.0001$, Poisson generalized linear model with a pairwise Tukey test) and wider compositional variability (i.e., dispersion) among conventional orchard than organically managed orchards (Fig. 3B; Adonis $R^2 = 0.145$, $P < 0.01$; betadisper $P < 0.05$). Our data also indicated that organic and conventional California orchards shared ten AMF taxa, including eight of the nine generalist taxa (Fig. 5). Those included three taxa belonging to the genus *Dominikia* (VTX00156, -304, -155), three to *Rhizophagus* (-092, -248, -100), two to *Septoglomus* (-63, -409), and two to unknown genera (-301, -214). One generalist taxon (unknown Glomeraceae VTX00323) and one specialist (*Funneliformis* VTX00067) were only associated with conventional orchards.

In contrast, several AMF taxa were unique to organic orchards including two *Septoglomus* (VTX00064, -331), one *Rhizophagus* (VTX00105), and three *Dominikia* (VTX130, -175, and 'Unknown'), among which *Dominikia* VTX00130 and *Septoglomus* VTX00064 were significantly enriched (Fig. 5: Wald's Test: $P < 0.01$).

HLB disease status significantly impacted AMF community, with a significant decline in observed richness of severely diseased trees compared to mild and moderately diseased (17.5 average ASVs in mildly diseased vs. 20.3 average ASVs in moderately diseased vs. 11.5 average ASVs in severely diseased trees; Fig. 2C; $P < 0.001$, Poisson generalized linear model with a pairwise Tukey test). Shifts in community composition was also seen as disease severity increased from mild to severe (Fig. 3C; Adonis $R^2 = 0.167$, $P < 0.05$; Adonis). Nine taxa were not detected in severely affected trees (VTX00075, -099, -113, -115, -125, -132, -146, -222, -323) (Fig. 6), of which, generalist unknown VTX00323 (Glomeraceae) and VTX00132 (*Dominikia*) were statistically supported (Fig. 6: Wald's Test: $P < 0.01$).

3.4 Discussion

Citrus is globally grown and an iconic crop to U.S. agriculture. Here, we deployed high throughput amplicon sequencing technology of the citrus root AMF to test whether community diversity and composition was affected by the climatic zones where citrus is grown (California and Florida), the type of farming system farming (organic vs. conventional) and the level HLB disease severity.

Our results indicate that commercial citrus trees in the U.S. are commonly found in association with AMF (78%), as reported in other systems (Smith and Read 2008; Brundrett and Tedersoo 2018) but AMF community composition in US orchards was more diverse than previously described. Nemec et al. (1981) used a morphological-based approach to identify AMF taxa inhabiting California and Florida citrus soils, and reported the cosmopolitan genus *Glomus*, as well as the two genera *Gigaspora* and *Sclerocystis*. Morphology and DNA sequencing-based approaches of orchards worldwide also reported that *Glomus* species were dominant AMF taxa including Brazil (Franca et al. 2007), China (Wang et al. 2012; Song et al. 2020) and Spain (Camprubí and Calvet 1996). In contrast, we found a broader number of AMF genera including *Dominikia, Funneliformis, Glomus, Rhizophagus, Septoglomus*, and likely additional undescribed genera within the Glomeraceae. Our results also indicated that *Dominikia* and *Rhizophagus* were the most abundant genera associated with citrus roots, although the *Dominikia* clade was not well supported. Higher taxonomic resolution beyond the genus level could not be achieved because of the short 220bp VT amplicon reads. A complete sequence of the WANDA/18S/ALM2 region will need to be obtained from a deeper sequencing run to identify distinct Glomeraceae species with strong branch support.

We achieved resolution of citrus-associated AMF clades by adopting a novel taxonomy assignment approach based on AMF-specific amplicons (Stefani et al. 2020). The disparity in the taxonomic profile between our results and previous reports has several reasons. In part, it can be explained by the recent taxonomic revision of the Glomeromycota phylum in which the family Glomeraceae was split into several families

(http://www.amf-phylogeny.com/; (Krüger et al. 2012)) and many *Glomus* species were moved to different genera. For example, the *Glomus* virtual taxa associated with citrus in China identified by Song et al. (Song et al. 2020) based on the MaarjAM database, clustered in our analysis with *Rhizophagus*, *Glomus*, *Septoglomus* and *Dominikia*. Furthermore, *Glomus fasciculatus* and *G. constrictus*, identified by Nemec et al. (1981) have now been renamed *Rhizophagus fasciculatus* and *Septoglomus constrictum*, respectively, and they associated with the clades of respective genera in our phylogenetic analysis. Our study also exclusively focused on root-associated AMF, but there have been reports of additional AMF taxa frequently occurring in bulk soil of citrus as reported by Nemec et al. (1981). These include *Gigaspora* spp. (Gigasporaceae) and *Glomus etunicatus* (now named *Claroideoglomus etunicatum*, Claroideoglomeraceae). Differential AMF composition may also have be driven by sampling size, seasonal and temporal variation (nearly 40 years between sampling events), edaphic properties of orchards, and varieties of citrus (Gao et al. 2019; Davison et al. 2021; Song et al. 2015).

The AMF community in Florida and California was composed of virtual taxa that were shared in both states while others were specific to each state. The seven most abundant and common virtual taxa within the genera *Dominikia* (VTX00156, VTX304), *Rhizophagus* (VTX00092 and VTX00248), *Septoglomus* (VTX00063) and unidentified Glomeraceae species (VTX00214 and VTX00301), were also associated with citrus in China (Song et al. 2020), apple in Italy (Turrini et al. 2017), and barrel medic (*Medicago truncatula*) in Tunisia (Mahmoudi et al. 2019) highlighting their cosmopolitan distribution. Both *Rhizophagus fasciculatus* and *Septoglomus constrictum* are known

generalist AM fungi capable of colonizing a broad range of soils (Oehl et al. 2010). In fact, *Rhizophagus* species have been used broadly in agriculture to improve soil, promote host plant growth, and cope with diseases (Ceballos et al. 2013; Pawlowski and Hartman 2020). Some VT classified as specialist taxa, VTX00113 and -115 (*Rhizophagus*) from Florida, were previously reported in Tunisia (Mahmoudi et al. 2019), reinforcing the need for a larger sampling size before to profile community structure. Nonetheless, these data support the view of a distribution of ubiquitous taxa across ecosystems (Öpik et al. 2009, 2010). The community segregation within the two distinct citrus climatic zones also indicates that community composition is driven by environmental conditions and ecological requirements of AMF (Öpik et al. 2013).

Our study also tested the impact of farming practices on AMF community richness and composition in citrus orchards. Farming practices and soil characteristics have been recognized to affect soil microbial biodiversity and fertility (Mäder et al. 2002; Hartmann et al. 2015). AMF are major components of soil agroecosystem structure, functionality, and productivity, and low input agricultural practices (e.g., organic farming) have been shown to be conducive to AMF biodiversity, activity, and root colonization in both annual (Oehl et al. 2003; Verbruggen et al. 2010) and perennial cropping systems (Franca et al. 2007; Turrini et al. 2017). Our results support those findings as we measured enriched AMF communities, including VTX00105 (*Rhizophagus*) and VTX00064 (*Septoglomus*), with distinct profiles in organic orchards in comparison to nearby conventional orchards. *Septoglomus constrictum* and *Rhizophagus intraradices* have been shown to stimulate plant growth or productivity in several cropping systems

and also to increase tolerance to heat and drought stress (Li et al. 2014; Ziane et al. 2017). However, AMF enrichment may also indicate a nutrient deficiency, and that the tree host needs to invest in the symbiotic relationship with AM fungi so that the cost in allocation of photo assimilated carbon is outweighed by the benefits of increased nutrient uptake (Graham et al. 1997; Johnson et al. 1997).

We found conclusive evidence of a significant depletion in AMF richness and shifts in community composition as HLB severity worsened, which corroborates previous findings (Ginnan et al. 2020). Trees affected by HLB expressed thin canopy, small leaves, and branch dieback symptoms and also showed significant root collapse. Root carbohydrate starvation is a result of carbon sequestration in the aboveground tissues and poor belowground translocation of photo assimilates as a result of phloem sieve tube occlusion from *C*Las infection (Etxeberria et al. 2009). This carbon source: sink unbalance of symptomatic trees is certainly affecting the rhizosphere microbiome and disturbing the symbiosis with AMF. Ginnan et al. (2020) measured enrichment of putative beneficial microbes in the early disease onset which is governed by changes in root signaling for the microbial recruitment events to occur (Bulgarelli et al., 2013). AMF are known to alter root exudate signaling and drive selection of certain microorganisms that would improve fitness under stress conditions (Jung et al. 2012) and may have contributed to the microbial recruitment efforts to protect its host. In the later stage of the disease, Ginnan et al. (2020) measured an enrichment with soilborne parasitic fungi and oomycetes (i.e., *Fusarium* and *Phytophthora*) and it was proposed to contribute to the root collapse and hasten the decline of trees impacted with HLB. Several studies have

highlighted the instrumental role of AMF in delaying disease onset or reducing symptoms against the soilborne pathogens *Fusarium* and *Phytophthora* in several pathosystems (Alaux et al. 2018; Wang et al. 2020) including citrus (Watanarojanaporn et al. 2011; Tian et al. 2021). Priming of plant immunity has been described as a major mechanism of AMF-induced disease resistance (Jung et al., 2012). The host defense response can be either localized in the roots or systemic throughout the plant and can be activated either constitutively or primed upon pathogen attack (Hohmann and Messmer 2017). The range of protection conferred by the symbiotic interaction depend on the ability of the AMF to control the plant host defense signaling pathway. Plants associated with AMF are more resistant to necrotrophic pathogens (*Fusarium* and *Phytophthora*) but more susceptible to biotrophs because they can activate jasmonic acid-mediated responses but need to repress salicylic acid-dependent ones (Jung et al., 2012). Given the plant defense benefits that AMF provide, practices that foster AMF diversity may result in extending tree longevity in the context of soil borne diseases and HLB.

In conclusion, this study is a first step to unravel the diversity and composition of AMF communities in citrus. In perennial cropping systems, growers rely on orchard longevity for profits and the root system is a major driver of tree health. Commercial application of mycorrhizae has gained traction as a farming practice, but success is limited to the host range of the AMF species used in the product formation and its biological fitness under agricultural constraints. Identifying beneficial generalist taxa that have potential for commercial application and developing recommendations for best cultural practices that support AMF diversity will help sustainable farming.

3.5 References

Alaux, P.-L., César, V., Naveau, F., Cranenbrouck, S., and Declerck, S. 2018. Impact of Rhizophagus irregularis MUCL 41833 on disease symptoms caused by Phytophthora infestans in potato grown under field conditions. Crop Prot. 107:26–33.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

Alvarado, P., Teixeira, M. de M., Andrews, L., Fernandez, A., Santander, G., Doyle, A., et al. 2018. Detection of Coccidioides posadasii from xerophytic environments in Venezuela reveals risk of naturally acquired coccidioidomycosis infections. Emerg. Microbes Infect. 7:1–13.

Berry, D., Ben Mahfoudh, K., Wagner, M., and Loy, A. 2012. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl. Environ. Microbiol. 78:612.

Boina, D. R., and Bloomquist, J. R. 2015. Chemical control of the Asian citrus psyllid and of huanglongbing disease in citrus. Pest Manag. Sci. 71:808–823.

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30:2114–2120.

Bové, J. M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. plant Pathol. :7–37.

Brundrett, M. C., and Tedersoo, L. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 220:1108–1115.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods. 13:581–583.

Camprubí, A., and Calvet, C. 1996. Isolation and screening of mycorrhizal fungi from citrus nurseries and orchards and inoculation studies. HortScience. 31:366–369.

Ceballos, I., Ruiz, M., Fernández, C., Peña, R., Rodríguez, A., and Sanders, I. R. 2013. The In Vitro Mass-Produced Model Mycorrhizal Fungus, Rhizophagus irregularis, Significantly Increases Yields of the Globally Important Food Security Crop Cassava. PLoS One. 8.

Chen, M., Arato, M., Borghi, L., Nouri, E., and Reinhardt, D. 2018. Beneficial services of arbuscular mycorrhizal fungi–from ecology to application. Front. Plant Sci. 9:1270.

Court, C. D., Hodges, A. W., Rahmani, M., and Spreen, T. 2018. Economic Contributions of the Florida Citrus Industry in 2015-16: FE1021, 7/2017. EDIS. 2018.

Davison, J., Moora, M., Öpik, M., Adholeya, A., Ainsaar, L., Bâ, A., et al. 2015. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. Science (80-.). 349.

Davison, J., Moora, M., Semchenko, M., Adenan, S. B., Ahmed, T., Akhmetzhanova, A. A., et al. 2021. Temperature and pH define the realised niche space of arbuscular mycorrhizal fungi. New Phytol. 231:763–776.

Dumbrell, A. J., Ashton, P. D., Aziz, N., Feng, G., Nelson, M., Dytham, C., et al. 2011. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytol. 190:794–804.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.

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.

Fan, J., Chen, C., Achor, D. S., Brlansky, R. H., Li, Z.-G., and Gmitter Jr, F. G. 2013. Differential anatomical responses of tolerant and susceptible citrus species to the infection of ‘Candidatus Liberibacter asiaticus.’ Physiol. Mol. Plant Pathol. 83:69–74.

Franca, S. C., Gomes-da-Costa, S. M., and Silveira, A. P. D. 2007. Microbial activity and arbuscular mycorrhizal fungal diversity in conventional and organic citrus orchards. Biol. Agric. Hortic. 25:91–102.

Gao, C., Montoya, L., Xu, L., Madera, M., Hollingsworth, J., Purdom, E., et al. 2019. Strong succession in arbuscular mycorrhizal fungal communities. ISME J. 13.

Gao, C., Montoya, L., Xu, L., Madera, M., Hollingsworth, J., Purdom, E., et al. 2019. Strong succession in arbuscular mycorrhizal fungal communities. ISME J. 13.

Ginnan, N. A., Dang, T., Bodaghi, S., Ruegger, P. M., McCollum, G., England, G., et al. 2020. Disease-induced microbial shifts in citrus indicate microbiome-derived responses to huanglongbing across the disease severity spectrum. Phytobiomes J. 4:375–387.

Glassman, S. I., Weihe, C., Li, J., Albright, M. B. N., Looby, C. I., Martiny, A. C., et al. 2018. Decomposition responses to climate depend on microbial community composition. Proc. Natl. Acad. Sci. 115:11994–11999.

Graham, J. H., Duncan, L. W., and Eissenstat, D. M. 1997. Carbohydrate allocation patterns in citrus genotypes as affected by phosphorus nutrition, mycorrhizal colonization and mycorrhizal dependency. New Phytol. 135:335–343.

Gu, Z., Eils, R., and Schlesner, M. 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 32:2847–2849.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., and Widmer, F. 2015. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 9:1177–1194.

Hervé, M. 2018. RVAideMemoire: testing and plotting procedures for biostatistics. R Packag. version 0.9-69. 3.

Hohmann, P., and Messmer, M. M. 2017. Breeding for mycorrhizal symbiosis: focus on disease resistance. Euphytica. 213:113.

Hothorn, T., Bretz, F., and Westfall, P. 2008. Simultaneous inference in general parametric models. Biometrical J. J. Math. Methods Biosci. 50:346–363.

Hu, J., Jiang, J., and Wang, N. 2018. Control of citrus Huanglongbing via trunk injection of plant defense activators and antibiotics. Phytopathology. 108:186–195.

Johnson, N. C., Graham, J. H., and Smith, F. A. 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. New Phytol. 135:575–585.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., and Pozo, M. J. 2012. Mycorrhiza-induced resistance and priming of plant defenses. J. Chem. Ecol. 38:651–664.

Krüger, M., Krüger, C., Walker, C., Stockinger, H., and Schüßler, A. 2012. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. New Phytol. 193:970–984.

Lee, J., Lee, S., and Young, J. P. W. 2008. Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol. Ecol. 65:339–349.

Lekberg, Y., Arnillas, C. A., Borer, E. T., Bullington, L. S., Fierer, N., Kennedy, P. G., et al. 2021. Nitrogen and phosphorus fertilization consistently favor pathogenic over mutualistic fungi in grassland soils. Nat. Commun. 12:1–8.

Lekberg, Y., Vasar, M., Bullington, L. S., Sepp, S.-K., Antunes, P. M., Bunn, R., et al. 2018. More bang for the buck? Can arbuscular mycorrhizal fungal communities be characterized adequately alongside other fungi using general fungal primers? New Phytol. 220:971–976.

Letunic, I., and Bork, P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47:W256–W259.

Li, S., Wu, F., Duan, Y., Singerman, A., and Guan, Z. 2020. Citrus greening: Management strategies and their economic impact. HortScience. 55:604–612.

Li, T., Lin, G., Zhang, X., Chen, Y., Zhang, S., and Chen, B. 2014. Relative importance of an arbuscular mycorrhizal fungus (Rhizophagus intraradices) and root hairs in plant drought tolerance. Mycorrhiza. 24:595–602.

Love, M. I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15.

Mäder, P., Fließbach, A., Dubois, D., Gunst, L., Fried, P., and Niggli, U. 2002. Soil fertility and biodiversity in organic farming. Science (80-.). 296.

Mahmoudi, N., Cruz, C., Mahdhi, M., Mars, M., and Caeiro, M. F. 2019. Arbuscular mycorrhizal fungi in soil, roots and rhizosphere of Medicago truncatula: diversity and heterogeneity under semi-arid conditions. PeerJ. 7:e6401.

McKenna, M. 2019. Antibiotics set to flood Florida's troubled orange orchards. Nature. 567:302–304.

McMurdie, P. J., and Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 8:e61217.

Miller, M. A., Pfeiffer, W., and Schwartz, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 gateway computing environments workshop (GCE)*, Ieee, p. 1–8.

Nemec, S., Menge, J. A., Platt, R. G., and Johnson, E. L. V. 1981. Vesicular: Arbuscular Mycorrhizal Fungi Associated with Citrus in Florida and California and Notes on Their Distribution and Ecology. Mycologia. 73.

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., et al. 2010. Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. Soil Biol. Biochem. 42:724–738.

Oehl, F., Sieverding, E., Ineichen, K., Mäder, P., Boller, T., and Wiemken, A. 2003. Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. Appl. Environ. Microbiol. 69:2816–2824.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. 2020. vegan: Community Ecology Package. R package version 2.5-6. 2019.

Öpik, M., Metsis, M., Daniell, T. J., Zobel, M., and Moora, M. 2009. Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest. New Phytol. 184:424–437.

Öpik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J. M., et al. 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytol. 188:223–241.

Öpik, M., Zobel, M., Cantero, J. J., Davison, J., Facelli, J. M., Hiiesalu, I., et al. 2013. Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza. 23:411–430.

Pawlowski, M. L., and Hartman, G. L. 2020. Reduction of Sudden death syndrome foliar symptoms and Fusarium virguliforme DNA in roots inoculated with rhizophagus intraradices. Plant Dis. 104:1415–1420.

Phillips, M. L., Weber, S. E., Andrews, L. V., Aronson, E. L., Allen, M. F., and Allen, E. B. 2019. Fungal community assembly in soils and roots under plant invasion and nitrogen deposition. Fungal Ecol. 40:107–117.

Smith, S., and Read, D. 2008. *Mycorrhizal Symbiosis*.

Song, F., Bai, F., Wang, J., Wu, L., Jiang, Y., and Pan, Z. 2020. Influence of citrus scion/rootstock genotypes on arbuscular mycorrhizal community composition under controlled environment condition. Plants. 9:1–16.

Song, F., Pan, Z., Bai, F., An, J., Liu, J., Guo, W., et al. 2015. The scion/rootstock genotypes and habitats affect arbuscular mycorrhizal fungal community in citrus. Front. Microbiol. 6.

Song, F., Pan, Z., Bai, F., An, J., Liu, J., Guo, W., et al. 2015. The scion/rootstock genotypes and habitats affect arbuscular mycorrhizal fungal community in citrus. Front. Microbiol. 6.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30:1312–1313.

Stefani, F., Bencherif, K., Sabourin, S., Hadj-Sahraoui, A. L., Banchini, C., Séguin, S., et al. 2020. Taxonomic assignment of arbuscular mycorrhizal fungi in an 18S metagenomic dataset: a case study with saltcedar (Tamarix aphylla). Mycorrhiza. 30:243–255.

Tian, L., Zou, Y.-N., Wu, Q.-S., and Kuča, K. 2021. Mycorrhiza-induced plant defence responses in trifoliate orange infected by Phytophthora parasitica. Acta Physiol. Plant. 43:1–8.

Turrini, A., Agnolucci, M., Palla, M., Tomé, E., Tagliavini, M., Scandellari, F., et al. 2017. Species diversity and community composition of native arbuscular mycorrhizal fungi in apple roots are affected by site and orchard management. Appl. Soil Ecol. 116:42–54 Available at: http://dx.doi.org/10.1016/j.apsoil.2017.03.016.

Verbruggen, E., Röling, W. F. M., Gamper, H. A., Kowalchuk, G. A., Verhoef, H. A., and van der Heijden, M. G. A. 2010. Positive effects of organic farming on below-ground mutualists: large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytol. 186:968–979 Available at: http://doi.wiley.com/10.1111/j.1469-8137.2010.03230.x.

Wang, P., Zhang, J. J., Shu, B., and Xia, R. X. 2012.   Arbuscular mycorrhizal fungi associated with citrus orchards under different types of soil management, southern China. Plant, Soil Environ. 58:302–308 Available at: http://www.agriculturejournals.cz/web/pse.htm?volume=58&firstPage=302&type=publishedArticle.

Wang, X., Ding, T., Li, Y., Guo, Y., Li, Y., and Duan, T. 2020. Dual inoculation of alfalfa (Medicago sativa L.) with Funnelliformis mosseae and Sinorhizobium medicae can reduce Fusarium wilt. J. Appl. Microbiol. 129:665–679.

Watanarojanaporn, N., Boonkerd, N., Wongkaew, S., Prommanop, P., and Teaumroong, N. 2011. Selection of arbuscular mycorrhizal fungi for citrus growth promotion and Phytophthora suppression. Sci. Hortic. (Amsterdam). 128:423–433.

Wickham, H. 2016. ggplot2-Elegant Graphics for Data Analysis. Springer International Publishing. Cham, Switz.

Wilson, G. W. T., Rice, C. W., Rillig, M. C., Springer, A., and Hartnett, D. C. 2009. Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecol. Lett. 12:452–461.

Wood, T. J., and Goulson, D. 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environ. Sci. Pollut. Res. 24:17285–17325.

Ziane, H., Meddad-Hamza, A., Beddiar, A., and Gianinazzi, S. 2017. Effects of arbuscular mycorrhizal fungi and fertilization levels on industrial tomato growth and production. Int. J. Agric. Biol. 19:341–347.

Table 3.1 Orchard location and varieties of citrus sampled indicating the number of root samples where AMF was found with the average number of Virtual Taxa.

Orchard#	US State	GPS Coordinates		Number of Root Samples Collected	Orchard Type	Scion Variety	Rootstock	Number of Root samples with AMF	Average Number of VT
1	Florida	27.027723	-80.485323	10	Conventional	Valencia	Swingle	9	12.9
2	Florida	27.446706	-80.325606	10	Conventional	Ruby Red	US897	9	8.56
3	Florida	28.981768	-81.924718	10	Conventional	Parson Brown	Swingle	9	25
4	Florida	28.714208	-81.774555	10	Conventional	Hamlin	Sour Orange	6	17.2
5	California	33.322922	-116.988861	8	Conventional	Newhall x Satsuma	Carrizo	7	7.43
6	California	36.353254	-119.059073	8	Conventional	Late Navel Powell	Carrizo	8	12
7	California	35.656439	-119.142610	8	Conventional	Late Navel Powell	Carrizo	2	16
8	California	33.322922	-116.988861	8	Conventional	Late Navel Powell	Carrizo	4	14.5
9	California	35.016722	-118.851070	8	Organic	Newhall x Satsuma	Carrizo	7	24.1
10	California	35.656066	-119.141074	8	Organic	Late Navel Powell	Carrizo	8	17.6

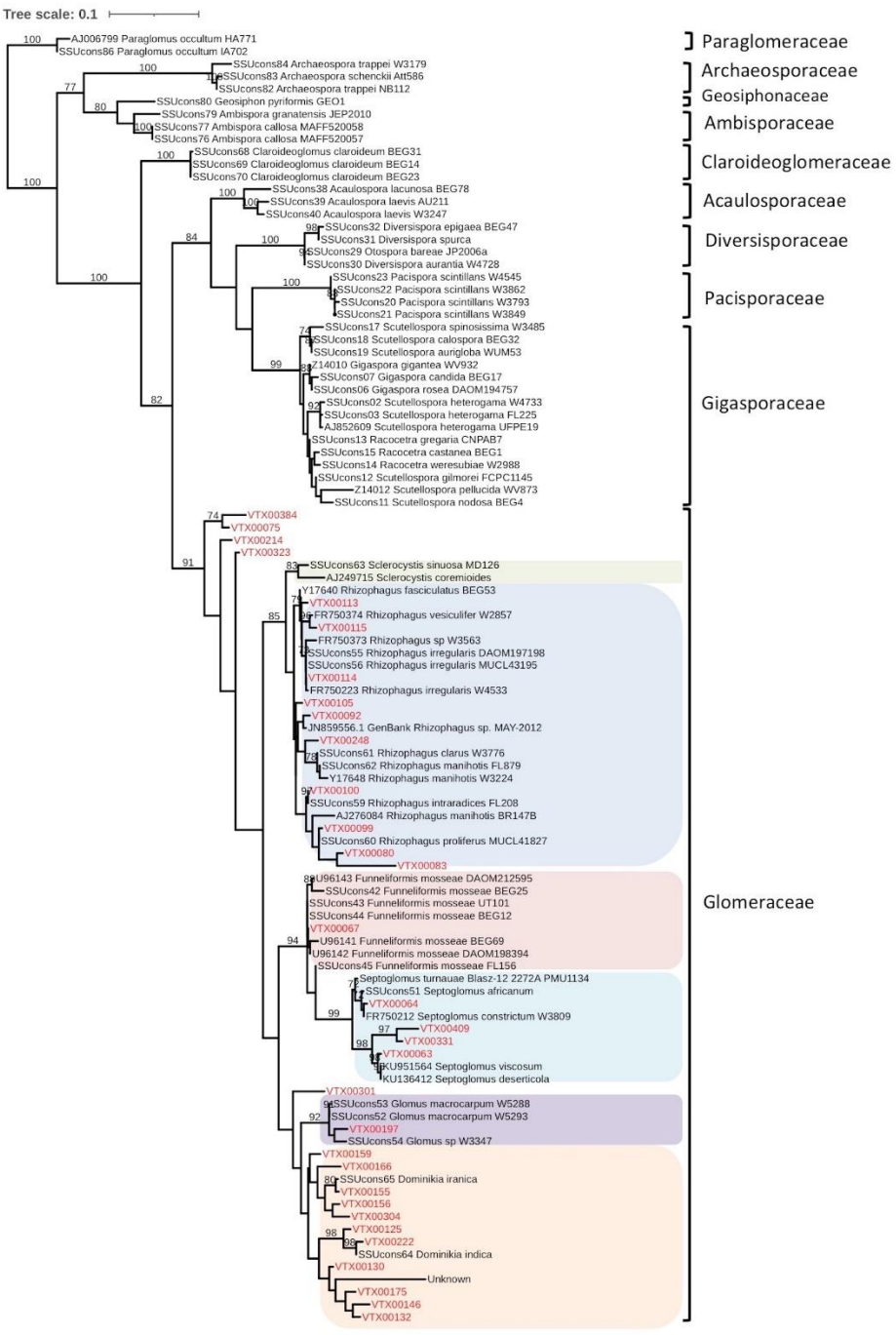

Figure 3.1 RAxML phylogenetic tree reconstructed by maximum likelihood analysis showing the genus taxonomy assignment of the 33 virtual taxa (in red). The tree represents 18 AMF genera and VT clustered with 5 colored AMF clades. Bootstrap values greater than 70 are displayed on the nodes.

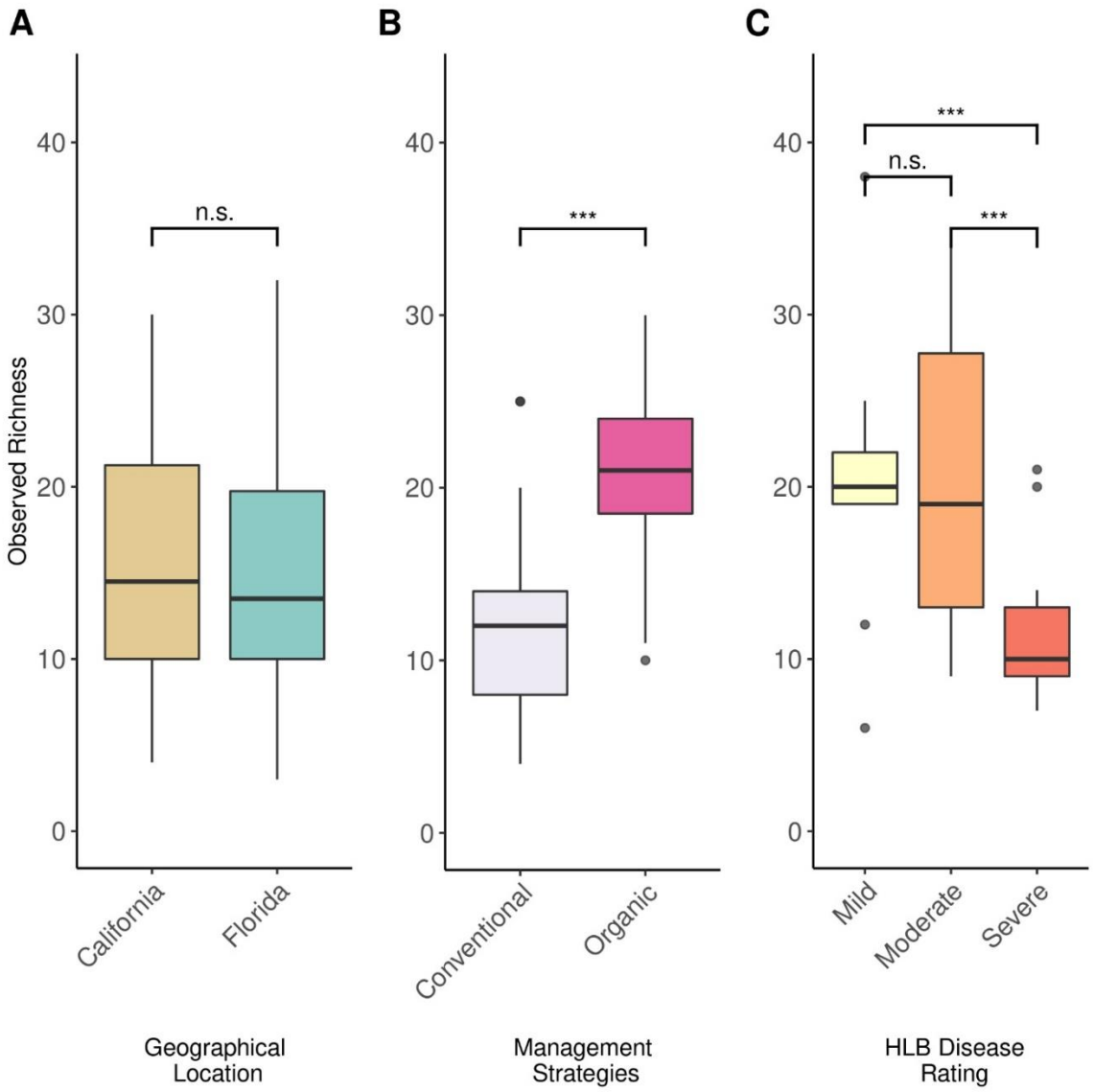

Figure 3.2 Alpha diversity plots comparing AMF richness across sample types; geographical location (A) shows no effect on richness, unlike management strategies (B) and HLB disease (C). Statistical significance is indicated for $P < 0.001$ (***) based on Poisson generalized linear model with a pairwise Tukey test. California n=36; Florida n=34; Conventional n=21; Organic n=15 and HLB symptom severity mild n= 12 trees; intermediate n= 10 trees; severe n= 11 trees.

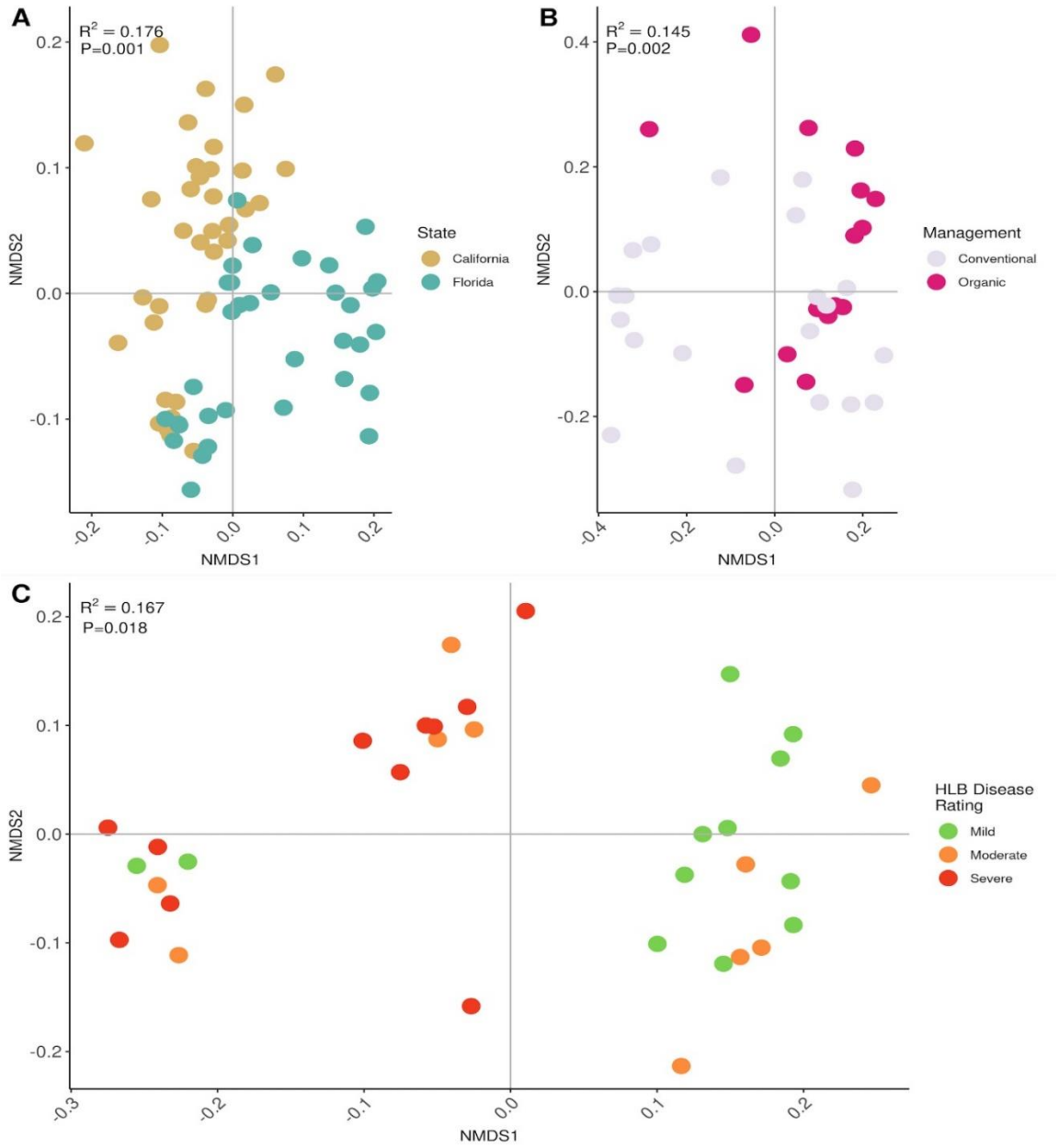

Figure 3.3 NMDS plots indicating that AMF beta diversity is significantly affected across sample types based on; (A) geographic location (A); management strategies in California (B); and HLB disease in Florida (C). Each dot represents the AMF community composition of a single tree. Points are colored by each group P-values and R^2 values were measured by permutational multivariate analysis of variance (Adonis) with values shown on the graphs.

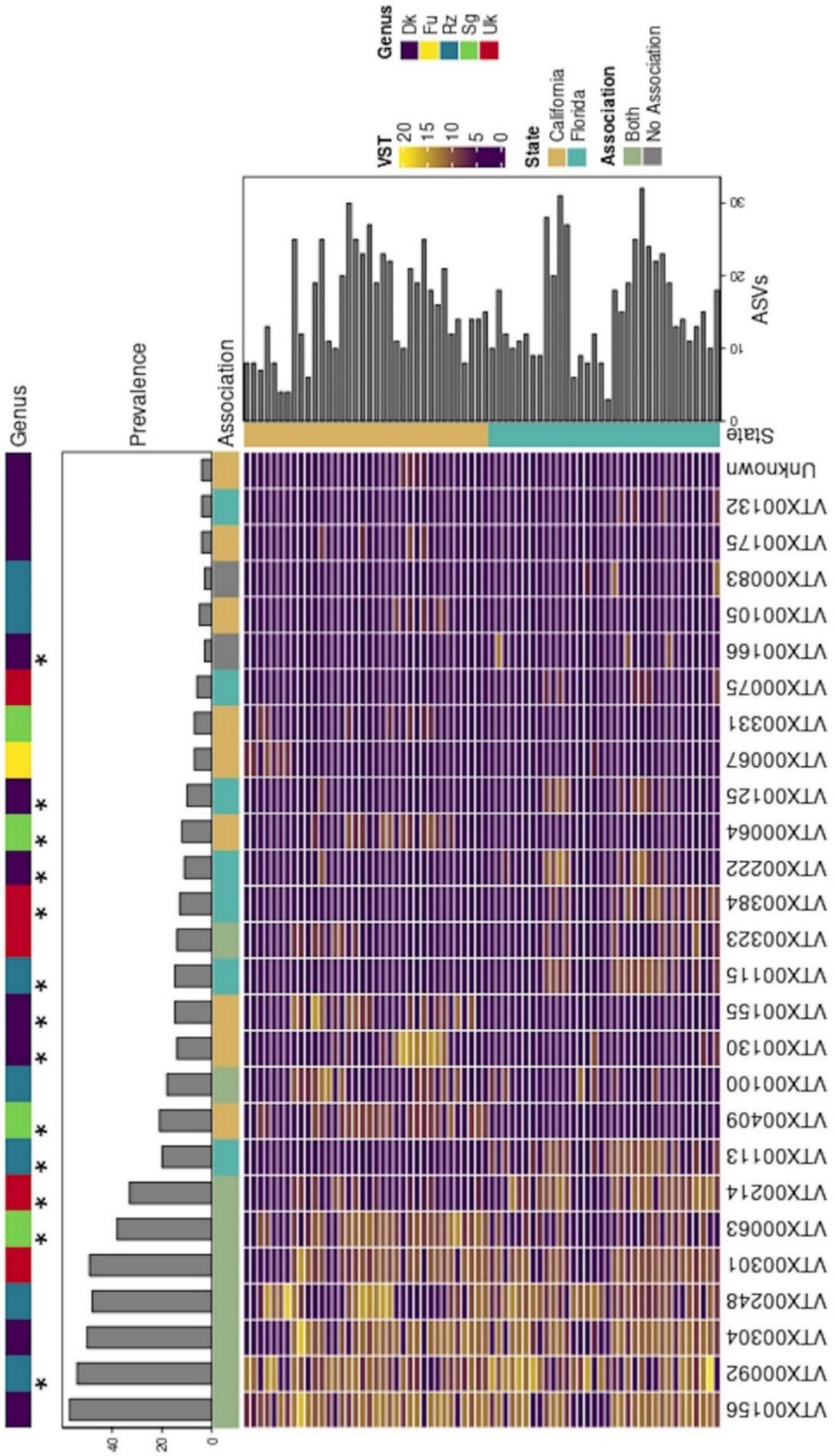
Genus
Prevalence
Association
State
ASVs
VST
Genus
Dk
Fu
Rz
Sg
Uk
State
California
Florida
Association
Both
No Association
VTX00156
VTX00092
VTX00304
VTX00248
VTX00301
VTX00063
VTX00214
VTX00113
VTX00409
VTX00100
VTX00130
VTX00155
VTX00115
VTX00323
VTX00384
VTX00222
VTX00064
VTX00125
VTX00067
VTX00331
VTX00075
VTX00166
VTX00105
VTX00083
VTX00175
VTX00132
Unknown

Figure 3.4 Heatmap of virtual taxa counts between geographical location following Deseq2 variance stabilization transformation. Each row represents a single root sample, and each column represents a unique virtual taxon. For clarity only virtual taxa that occurred in three or more samples are displayed. Top column annotation depicts the genus to which the virtual taxa clustered with based on Maximum likelihood tree in figure 1. Asterisk (*) depict virtual taxa differentially significantly abundant between geographical location per DESeq2 Wald's test. Column annotation bar graph depicts the prevalence (number of unique samples) the virtual taxa were found in. The "Association" column annotation is a colorimetric Venn diagram with a 10% prevalence cut-off where yellow squares represent taxa associated with California samples, blue squares represent taxa associated with Florida samples and green squares represent taxa which were associated with both Florida and California citrus roots. Gray boxes indicate taxa that did not pass the 10% prevalent cut-off to be associated with either category. Row annotations show which samples belong to each geographical location and bar graph shows the number of unique ASVs associated with each sample. Dk: *Dominikia*; Fu: *Funneliformis*; Rz: *Rhizophagus*; Sg: *Septoglomus*; Uk: Unknown.

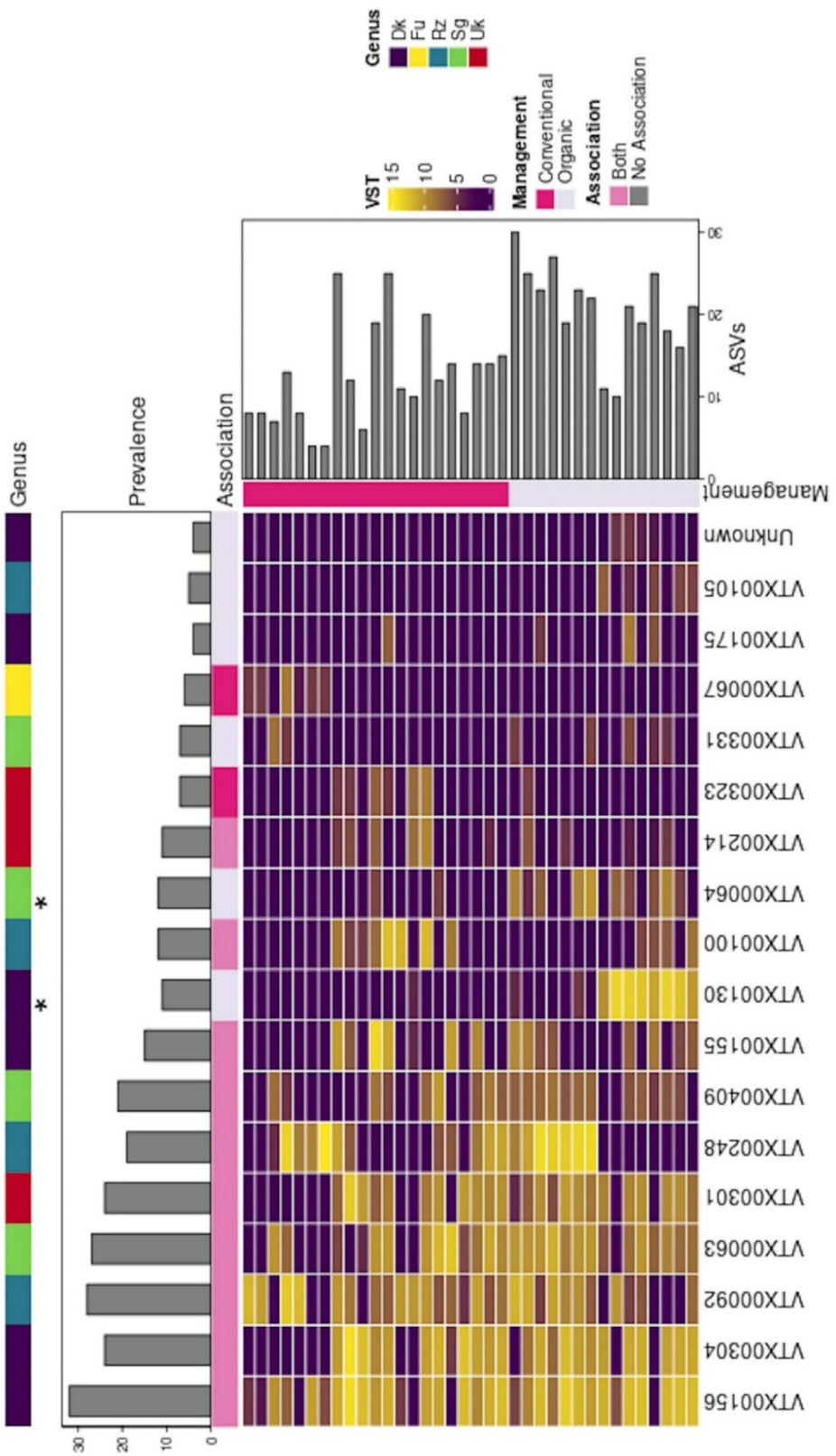
Genus
Dk
Fu
Rz
Sg
Uk
VST
15
10
5
0
Management
Conventional
Organic
Association
Both
No Association
ASVs
30
20
10
0
Management
Association
Prevalence
30
20
10
0
Genus
*
*
VTX00156
VTX00304
VTX00092
VTX00063
VTX00301
VTX00248
VTX00409
VTX00155
VTX00130
VTX00100
VTX00064
VTX00214
VTX00323
VTX00331
VTX00067
VTX00175
VTX00105
Unknown

Figure 3.5 Heatmap of virtual taxa counts between management strategy from California samples following Deseq2 variance stabilization transformation. Each row represents a single root sample, and each column represents a unique virtual taxon. For clarity only virtual taxa that occurred in three or more samples are displayed. Top column annotation depicts the genus to which the virtual taxa clustered with based on Maximum likelihood tree in figure 1. Asterisk (*) depict virtual taxa differentially abundant between geographical location per DESeq2 Wald's test. Column annotation bar graph depicts the prevalence (number of unique samples) the virtual taxa were found in. The "Association" column annotation is a colorimetric Venn diagram with a 10% prevalence cut-off where dark pink squares represent taxa associated with Conventional samples, lavender squares represent taxa associated with Organic samples and medium pink squares represent taxa which were associated with both Organic and Conventional citrus roots. Row annotations show which samples belong to which management strategy and bar graph shows the number of unique ASVs associated with each sample. Dk: *Dominikia*; Fu: *Funneliformis*; Rz: *Rhizophagus*; Sg: *Septoglomus*; Uk: Unknown.

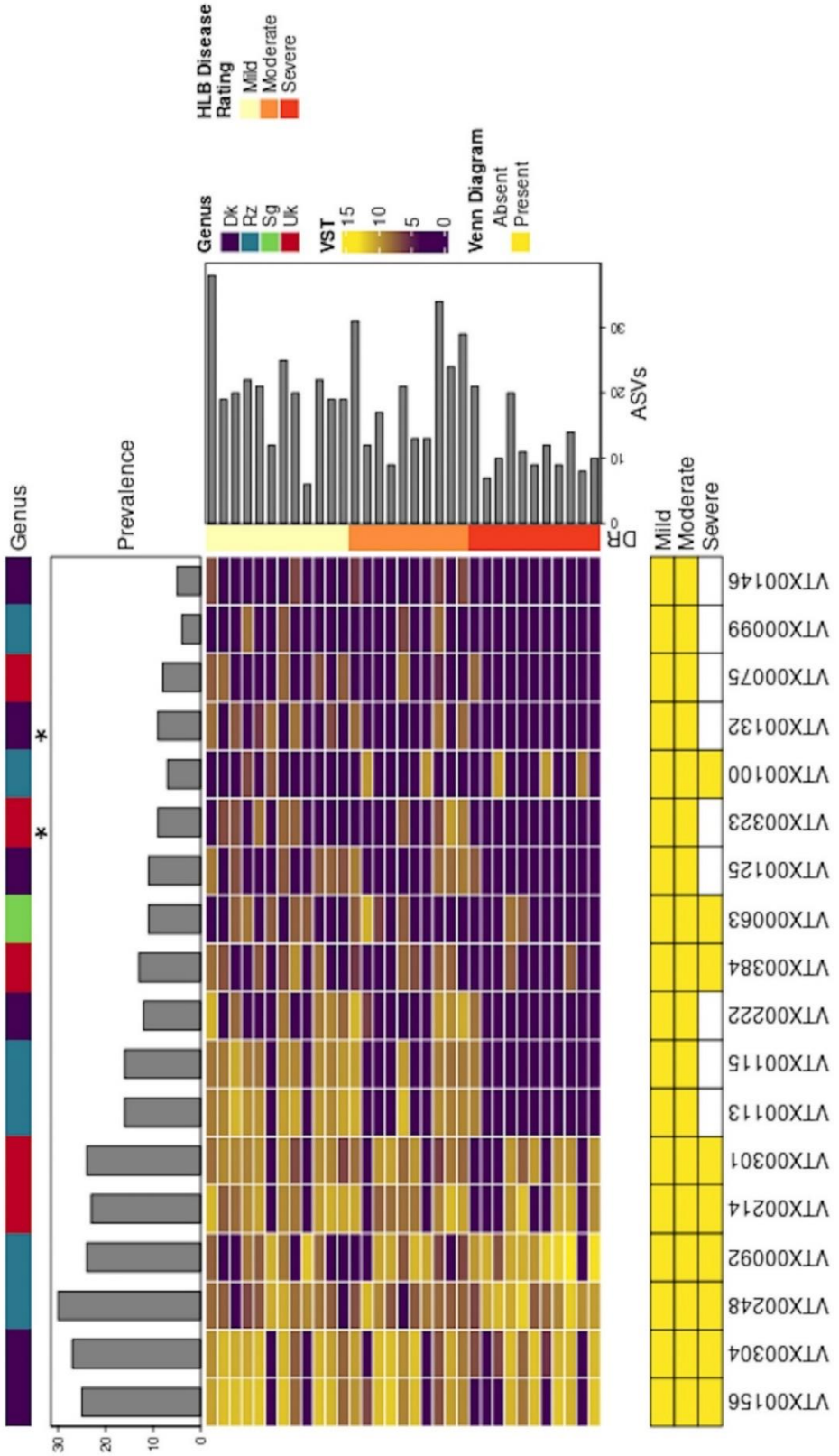
HLB Disease Rating
Mild
Moderate
Severe
Genus
Dk
Rz
Sg
Uk
VST
15
10
5
0
Venn Diagram
Absent
Present
Genus
Prevalence
ASVs
D3
Mild
Moderate
Severe
VTX00156
VTX00304
VTX00248
VTX00092
VTX00214
VTX00301
VTX00113
VTX00115
VTX00222
VTX00384
VTX00063
VTX00125
VTX00323
VTX00100
VTX00132
VTX00075
VTX00099
VTX00146

Figure 3.6 Heatmap of virtual taxa counts of HLB disease severity from Florida samples following Deseq2 variance stabilization transformation. Each row represents a single root sample, and each column represents a unique virtual taxon. For clarity only virtual taxa that occurred in three or more samples are displayed. Top column annotation depicts the genus to which the virtual taxa clustered with based on Maximum likelihood tree in figure 1. Asterisk (*) depict virtual taxa differentially abundant between geographical location per DESeq2 Wald's test. Column annotation bar graph depicts the prevalence (number of unique samples) the virtual taxa were found in. Row annotations show which samples belong to each disease rating severity and bar graph shows the number of unique ASVs associated with each sample. Venn diagram heatmap with yellow rectangles show which taxa were found in at least 10% of sample from each disease severity. Dk: *Dominikia*; Rz: *Rhizophagus*; Sg: *Septoglomus*; Uk: Unknown.

4 Bioassay to Evaluate Plant Growth-Promoting Rhizobacteria (PGPR) in Citrus

4.1 Introduction

Global population expansion, food waste, and limitation of available resources are all current agricultural challenges (Velten et al. 2015). The Green Revolution, which began in the late 20th century, greatly increased plant productivity and crop yields by introducing new high-yielding seed varieties and increasing the use of synthetic fertilizers, insecticides, and other agrochemicals (Kesavan and Swaminathan 2018). However, over the past few decades, there has been a global decline in the biological and physicochemical health of the arable soil due to the rampant usage of synthetic agrochemicals (Bishnoi 2018; Pingali 2012). An effective strategy to stop the rapid environmental deterioration while securing long-term global food supply is to promote sustainable agriculture with a gradual decrease in the use of synthetic agrochemicals and a more prominent utilization of the biological and genetic potential of microorganisms (Liu et al. 2016).

Plant growth-promoting rhizobacteria (PGPR) inhabit the rhizosphere, where they account for 5 to 17% of total root surface area (Gray and Smith 2005). These microorganisms have positive effects on plant growth, seed germination, and seedling emergence (Ahmad et al., 2008). Ortíz-Castro et al. (2009) hypothesized that PGPR strains can stimulate plant growth and development both directly and indirectly. Direct mechanisms affect the balance of plant growth regulators and induce the plant's

metabolism, resulting in an increase in its adaptive capacity. These includes nitrogen fixation, production of phytohormones, solubilization of minerals, production of siderophores and enzymes, and induction of systemic resistance. In contrast, indirect mechanisms necessitate the involvement of the plant's defense metabolic systems, which respond to the signal supplied by the bacterium impacting the plant (Goswami et al. 2016). These include antibiotic production, iron chelation, and synthesis of extracellular enzymes to hydrolyze the fungal cell wall (Zahir et al. 2004; Van Loon 2007). The global use of PGPR has increased significantly during the past decade but its market size remains negligible in comparison to synthetic agrochemicals (Timmusk et al. 2017; Soumare et al. 2020). The agrochemicals market size was estimated at over $ 238 billion in 2018 and is anticipated to reach $328 billion by 2026. In comparison, the global biofertilizer industry is anticipated to reach $3.5 billion by 2025 (Soumare et al. 2020). The biopesticides market globally was valued at $3 billion in 2018, accounting for just 5% of the total crop protection market (Damalas and Koutroubas 2018). Examples of microbes that have been formulated into commercial products include; (i) the nitrogen-fixing bacteria Rhizobium and Azotobacter; (ii) phosphate solubilizer bacteria Bacillus and Pseudomonas and arbuscular mycorrhizal fungi (AMF) Rhizophagus and Glomus; and (iii) the biocontrol bacteria Streptomyces and fungi Trichoderma (García-Fraile et al. 2017).

The citrus fruit is one of the most economically significant crops and is grown and sold all over the world. Several varieties of citrus, including grapefruit, lemon, lime, orange, tangerine, and numerous hybrids, are produced in California. There has been a

large emphasis on mining the citrus microbiome to find solutions to some of the pressing issues in global citrus production, and specifically the management of Huanglongbing disease (Ginnan et al., 2018, 2020; Xu et al., 2018; Zhang et al., 2021). It has been reported that several bacteria isolated from citrus (Paenibacillus validus, Lysinibacillus fusiformis, Bacillus licheniformis, Pseudomonas putida, Microbacterium oleivorans, and Serratia plymutica) could enhance plant growth and reduce HLB symptoms (Trivedi et al. 2011). In addition, arbuscular mycorrhizal fungi are commonly found in association with healthy trees and could provide some level of protection against HLB and root rot diseases (Xi et al. 2022). It has been also reported that several bacterial taxa (Bacillus, Enterobacter, Pseudomonas) promoted the growth of citrus seedlings (Giassi et al. 2016; Mushtaq et al. 2019; Thokchom et al. 2014).

Public awareness of environmental risks has expanded consumer demand for organic or sustainably grown food products which, in turn, shifted the standard conventional farming practices to more integrated systems. The use of agricultural biological products has become an integrated part of pest and disease management practices and nutritional programs in developed markets. However, the lack of consistency of bioproduct efficacy in comparison so synthetic agrochemicals has limited its broad adoption among growers. This is in part due to the intricate relationship between the microbe and the plant and that most bioinoculants have a specific host range. In addition, bioinoculants may only work under optimal environmental conditions that are often not met in agroecosystems. Here we establish the foundation using a citrus seedling bioassay that enable the evaluation of commercial bioinoculants and citrus-associated

microbes as plant growth promoters. This work will lead to a broader adoption of commercial bioproduct among citrus nursery and orchard production managers and will facilitate the development of new biotechnology that are targeted to citriculture

4.2 Material and Method

In Planta Selection and Evaluation of PGPR on Citrus Growth

Seeds of ‘Carrizo’ citrange (Citrus sinensis 'Washington' sweet orange X Poncirus trifoliata) (Lyn Citrus Seed, Bakersfield, CA) were used in this study. Before planting, partial seeds had been stored in fridge around one year and would be referred as ‘old seeds’; other seeds that were freshly collected referred as ‘fresh seeds’ in this study. All seeds were planted in SC10-type plastic cone-tainers (Stuewe and Sons., Tangent, OR) containing 100 g of autoclaved sand: vermiculite = 1: 1 (v/v) with 10 NPK 14-14-14 beads (Scotts Osmocote Classic). The experiment was conducted in a growth chamber (HiPoint FH-2300 Environmental Chamber, Taiwan) in randomized block design with the following conditions: temperature 30 °C, 16 hours photoperiod with the light intensity of 500 μmol/m2·s-1 (RGB:50,50,50), relative air humidity 60% during the day and 50% during the night.

The initial stage of this study consisted of a seed germination assessment on old seeds of 6 bacterial isolates (all non-commercial isolates in this study were collected as described in (Blacutt et al. 2020)): two *Bacillus* spp. isolates: CB902, CB912 , Pantoea spp. isolates CB072 , Curtobacterium spp. isolates CB892 (these isolates were obtained

from citrus grove soil in Florida), one commercial *Streptomyces* product Actinovate (*Streptomyces lydicus* WYEC108) (A.M. Leonard, Piqua, OH) and one commercial *Bacillus* product Serenade (*Bacillus subtilis* strain QST713) (stated as *Bacillus* SER) (Bayer AG, Leverkusen, Germany) were tested for seed germination rate and bacterial restoration rate. Each seed received 10^3 cell per gram of soil with one of 6 treatments or same amount of sterile 1x PBS (phosphate buffered saline) as control group. Bacteria were grown in Petri dishes containing Tryptic Soy Agar (TSA) (BD Difco™, Becton Dickinson and Company, NJ) and incubated at 28 °C for 48 h. Pure culture colony was picked from Petri dishes and grown in 13 mm test tube containing Tryptic Soy Broth (TSB) (BD Difco™, Becton Dickinson and Company, NJ) and incubated at 28 °C for 24 h. To prepare each inoculum, the colonies were diluted with sterile PBS to 10^5 cells/mL as inoculum. A 2-mL aliquot of suspension of each bacterial isolate was added to each cone, ended up a final concentration of 10^3 CFU per gram of soil. Each treatment was replicated 20 times, leading to a total of 140 cones of citrus seedlings. These seedlings were watered 3 times a week for the duration of the experiment using autoclaved DI (deionized) water. No additional nutrients were added to the soil during the experiment. This experiment has been repeated once to confirm consistency.

After the assessment of bacterium on promoting seed germination, seven bacterial isolates or products were selected or added for experiment exploring plant growth promotion traits on fresh seeds, including one *Pseudomonas* spp. isolates CB204, one *Rhizobium* spp. isolates CB690, one *Burkholderia* spp. isolates CB691, one commercial *Streptomyces* product Actinovate (A.M. Leonard, Piqua, OH), and three *Bacillus* spp.

CB729, CB902, and *Bacillus* SER. Bacterium inoculation and plant maintenance were same as the old seed experiment except for each treatment was replicated 16 times, leading to a total of 128 cones of citrus seedlings. This experiment was carried out in duplicate. Data collected from both rounds were combined and presented.

The following parameters of interest were surveyed or measured every week during the growth of the seedlings: germination rate (%), plant height (cm), and leaf number. Plants were harvested after 50 days. Shoot and root fresh weight were measured when harvesting.

Rhizosphere Microbe Isolation

The soil associated with roots was collected and denoted as the rhizosphere fraction. When harvesting, seedling was carefully taken out from the cone, excess soil was gently shaken off and root was sealed into a new clean ziploc bag (SC Johnson., US). The bag was shaken well for 15 seconds to let the rhizosphere fall apart from the root, then the rhizosphere was collected into a 2 mL Eppendorf tube. To acquire the microbes, 1 mL of sterile PBS was added, and the tube was incubated at 28 C, 150 rpm for one hour. After the incubation, 100 µL of supernatant (diluted 100 times with sterile PBS) was plated into a Petri dish containing TSA. These plates were incubated at 28 C for 24 h.

Root Endophytes Isolation

The shoot and root of each seedling was cut apart using sterile scissors, the root was surface sterilized by immersing in 70% ethanol for 30 sec, in 20% bleach for 30 sec, 1

min in sterile water, and 1 min in another sterile water. The clean root was transferred into a new meshed sample bag (Agdia, Inc., IN), 2 mL of PBS was added into the bag at same time, then the root was smashed until all tissue was lysed (about 5 seconds). Then 1 mL of the root slurry was collected into a 1.7 mL Eppendorf tube and 100 µL of supernatant was plated into a Petri dish containing TSA. These plates were incubated at 28 C for 24 h.

PCR Amplification and Sequencing

The number of bacterial colonies on TSA medium were recorded identity of bacteria recovered from soil and root isolations was confirmed by sequencing of the 16S rDNA regions using the pair of primers 799 f (5'-AACMGGATTAGATACCCKG-3') (Chelius and Triplett 2001) and 1492 r (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). PCR reaction contained 1 µL of DNA, 0.5 µL of each primer (10 mM), 10 µL of Phusion hot start flex 2× master mix and 13 µL of DNA-/RNA-free water, leading to a total reaction volume of 25 µL. After initial denaturation at 94 °C for 5 min, each thermal cycling was as follows: denaturation at 94 °C for 1 min, annealing at 52 °C for 45 s, and elongation at 72 °C for 1 min. At the end of 30 cycles, the final extension step was at 72 °C for 8 min. Results were checked on a 1% agarose gel and purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The purified DNA was sent for Sanger sequencing at the UC Riverside Genomics Core facility. All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and the

16S rDNA similarity sequences searches were performed using the BLASTN tool in the NCBI website.

Statistical Analysis and Data Visualization

Data collected from both rounds were combined and presented. *Chi*-squared test was used to compare treatment germination rate to control rate. Pairwise student t-test ($p < 0.05$) was used to compare treatment means with control means. All the statistical analyses were carried out using R v4.1.1 (R Core Team, 2021). All data were visualized using SigmaPlot 14.0 (Systat Software, Erkrath, Germany).

4.3 Result

The evaluation of bacterial isolates' capacity to promote germination of old Carrizo rootstock seeds indicated that all three Bacillus isolates treatments induced a significant increase in seed germination rate, with a 100%, 117%, 133% increase for treatments with Bacillus SER (Chi-squared test; $p = 0.064$), Bacillus 912 (Chi-squared test; $p = 0.0326$), and Bacillus 902 (Chi-squared test; $p = 0.0232$) compared to the controls, respectively. No significant difference was measured for Pantoea 072, Curtobacterium 892 and commercial Streptomyces ACT. When we repeated the experiment with fresh seed, the mean germination rate for all treatment groups was at 92%, ranging from 84% for Pseudomonas to 100% for Burkholderia, with no significant differences in relation to the untreated controls (Fig. 1).

Assessment of the ability of bacterial isolates to promote growth of Carrizo using fresh seed revealed that Bacillus SER, Bacillus 902 and Burkholderia 691 significantly increase plant height by 27.1% 17.9% and 16.9% relative to the controls, respectively (student t-test; $p < 0.001$, $p = 0.014$, $p = 0.013$, respectively). Furthermore, all treatments that significantly increased plant height also significantly increased the total number of leaves (Fig. 2) and shoot fresh weight, except for Burkholderia 691. Hence, Bacillus SER, Rhizobium 690, and Bacillus 902 increased fresh shoot weight by 36.5%, 27.3%, 24.9% relative to the controls, respectively (student t-test; $p = 0.006$, $p = 0.008$, $p = 0.024$, respectively). Streptomyces ACT was the only treatment showing a significant increase in total number of leaves (student t-test; $p = 0.015$) and shoot fresh weight (25.5%) (student t-test; $p = 0.027$) that did not have a significant effect on plant height. Pseudomonas 204, Streptomyces ACT, and Bacillus 729 showed no effects on shoot growth and weight. Interestingly, the positive effects of those treatments on above ground plant growth did not translate to root biomass. Surprisingly, Burkholderia 691. and Bacillus 729 induced significantly decrease by 20.2% (student t-test; $p = 0.045$) and 20.7% (student t-test; $p = 0.049$) in root fresh weight in relation to controls, respectively (Fig. 3). In addition, seeds treated with Rhizobium 690 and Burkholderia 691 showed a similar phenotype with a significant lower root:shoot ratio (Fig.4). When comparing microbial recovery rates, all treatments with Bacillus isolates had a 100% recovery rate of the bacterium (Fig. 5). Bacterial recovery rate from plants was average for Burkholderia 691 (50%), Pseudomonas 204 (50%), Rhizobium 690 (60%), and low for Streptomyces ACT (20%).

4.4 Discussion

In today's quest for a sustainable agriculture, PGPR is a promising solution because they exhibit synergistic and antagonistic interactions with the soil microbiota and engage in an array of activities of ecological significance (Basu et al. 2021). In this study, we investigated the role of PGPR in promoting citrus seed growth and development. Results from our experiments indicated that our bioassay was quick and reliable because we collected reproducible data within a 50-day period.

In the seed germination experiment we measured a quick drop of the germination as Carrizo seeds aged. Reactive oxygen species (ROS) are believed to be the primary cause of seed aging and decrease in germination rates. ROS damages cell membrane phospholipids and causes structural and functional deterioration of proteins and genetic material, and especially the degradation of catalase, an enzyme that protect against oxidative damage cause by ROS (Orbović et al. 2013; Rajjou et al. 2012). Studies have also measured dramatic declines in phytohormone levels in 3-month old Vellozia alata seeds, including gibberellins, abscisic acid, cytokinins, and jasmonic acid (Munne-Bosch et al. 2011). Phytohormone levels change dramatically during seed germination. According to the hormone balance theory, the ratio of abscisic acid (ABA) gibberellic acid (GA) serves as the primary determinant of seed dormancy and germination (Taiz et al. 2015), whereby ABA exerts an inhibitory effect and GA a positive effect on seed germination. Other phytohormones like cytokinin, auxin (IAA), ethylene and brassinosteroids form a signaling network that also affect germination, particularly in

response to environmental constraints (Subbiah and Reddy 2010; Belin et al. 2009; Rajjou et al. 2012). Several reports have indicated that in citrus GA increases germination rates in sweet orange (Burns and Coggins 1969), Cleopatra mandarin and sour orange (Abou-Rawash et al. 1980). Our results showed that all the treatments with Bacillus isolates stimulated seed germination. PGPR have been shown to stimulate seed germination in several systems including citrus (Thokchom et al. 2014; Swain et al. 2007), likely because of their ability to produce phytohormones (Glick et al. 1999). Bacillus is well known phytohormone producer, and B. pumilus and B. licheniformis have been documented to be capable of producing GAs (Gutiérrez-Mañero et al. 2001) and B. subtilis IAA (Swain et al. 2007). A sequencing and annotation of the Bacillus genomes and measurement of phytohormone level production in vitro will provide qualitative profiling of the phytohormones.

We measured striking differences in bacterial root colonization rates in our experiment ranging from complete colonization with Bacillus to poor colonization with Streptomyces. The relationship between the plant and its surrounding microbes is intricate and plants recruit microbes to fulfill specific biological functions. Root exudates act as signal molecules and food sources for the selective recruitment of microbes from bulk soil in exchange of increased nutrients assimilation and improved tolerance pathogens (Compant et al. 2010). Streptomyces is known to produce an array of antimicrobial compounds (Jia et al. 2017) and has been utilized in agriculture as a biocontrol agent and its benefits are mostly observed when introduced in a pathosystem (Yuan and Crawford 1995). In our study, we did not challenge citrus with a pathogen

which may explain the lack of root colonization and beneficial outcome on the host. In contrast, Bacillus has been reported as a very efficient root colonizer and forms aggregates or microcolonies on the surface of plant roots after inoculation (Trivedi et al. 2005; Khalid et al. 2004; Zhang et al. 2011). We show that positive effects on plant growth (plant height, shoot weight, leaf numbers) were consistently measured and with a strong significance when Bacillus treatments were used. Similar outcomes were obtained with yams seeds were treated with Bacillus subtilis and showed an increased in both roots and shoots length and biomass (Swain et al., 2007). In a recent study, transcriptomic analysis of cucumber roots in response to a commercial Bacillus SER indicated that plant genes involved in phytohormone production and nutrient availability were upregulated suggesting that these mechanisms could be involved in citrus growth promotion (Samaras et al. 2022). The role of Bacillus on exogenous production of phytohormone for seed germination has already been discussed and similar mechanisms could also apply to plant tissue growth and development. In addition, Bacillus has been described as a diazotrophic bacterium capable of biological N fixation (BNF) both in the rhizosphere and inside the roots of several agricultural crops (Puri et al. 2018; Seldin et al. 1984; von der Weid et al. 2002) although to our knowledge it has not been described in citrus. Diazotrophic bacteria fix nitrogen by using carbon and energy sources supplied from the root environment, and the bacteria release fixed N probably after lysis of the bacterial cells. The associations of plants and microbes in rhizosphere soils and plant root endosphere form adapted nitrogen-fixing systems under physiologically nitrogen-deficient but energy-sufficient conditions (Mahmud et al. 2020). Bacillus have also been identified as

phosphorous solubilizing microorganisms (PSM) capable of transforming insoluble phosphorus in soils to soluble forms can function as biofertilizers for plants (Alori et al. 2017).

Similar to Bacillus, Burkholderia and Rhizobium are categorized as both diazotrophic and phosphorous solubilizing bacteria (Alori et al. 2017; Puri et al. 2018). They stimulated plant growth but unlike Bacillus they induced a significantly lower root: shoot ratio (RSR). RSR is a measurement of the amount of plant tissues with supportive functions (roots) compared to the amount of plant tissue with growth function (shoots). It is governed by a functional balance between root uptake of water and nutrient and leaf photosynthesis. Nutrient acquisition and uptake are regulated to optimize nutrients needed for growth and reproduction (Thomas 2016). According to the concept of functional equilibrium, under nutrient-deficient conditions such as low N or P, the root: shoot ratio increases to enhance soil exploration and nutrient uptake (Brouwer 1983). Thus, the low RSR observed with Rhizobium and Burkholderia treatments indicate that seedlings are not under nutrient deficiency and as such tend to allocate more resources to shoot. One possible explanation is that Rhizobium and Burkholderia promoted the citrus by providing sufficient acquisition of nitrogen and phosphorous causing the partition of the plant biomass to aboveground.

In summary, the bioassay developed on Carrizo citrange seed allow for a rapid and reliable screening of PGPR. Commercial and wild isolates of Bacillus affected seed germination and overall seedlings growth and likely because of phytohormones

production. We hypothesize that Rhizobium and Burkholderia promote root nutrient acquisition in the form of N and P as indicated by a low RSR. In future works, genome sequencing of these isolates will allow for naming of PGPR species and genome annotation will reveal clues about potential mechanisms of action. Plant transcriptomics will determine which genes are upregulated in the presence of PGPR and will better characterize the nature of the plant microbe interaction.

4.5 Reference

Abou-Rawash, M., Montaser, A., El-Nabawy, S., Mahmoud, N., and Habib, S. S. 1980. Germination of some citrus seeds as affected by soaking [soaking cleopatra mandarin and sour orange seeds] in growth regulators, water washing and sowing date. Res. Bull. Fac. Agric. Ain Shams Univ., Cairo.

Alori, E. T., Glick, B. R., and Babalola, O. O. 2017. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front. Microbiol. 8:971.

Basu, A., Prasad, P., Das, S. N., Kalam, S., Sayyed, R. Z., Reddy, M. S., et al. 2021. Plant growth promoting rhizobacteria (PGPR) as green bioinoculants: recent developments, constraints, and prospects. Sustainability. 13:1140.

Belin, C., Megies, C., Hauserova, E., and Lopez-Molina, L. 2009. Abscisic acid represses growth of the Arabidopsis embryonic axis after germination by enhancing auxin signaling. Plant Cell. 21:2253–2268.

Bishnoi, U. 2018. Agriculture and the dark side of chemical fertilizers. Environ. Anal. Ecol. Stud. 3.

Blacutt, A., Ginnan, N., Dang, T., Bodaghi, S., Vidalakis, G., Ruegger, P., et al. 2020. An in vitro pipeline for screening and selection of citrus-Associated microbiota with potential anti-"Candidatus liberibacter asiaticus" properties. Appl. Environ. Microbiol. 86:1–18.

Brouwer, R. 1983. Functional equilibrium: sense or nonsense? Netherlands J. Agric. Sci. 31:335–348.

Burns, R., and Coggins, C. 1969. Sweet orange germination and growth aided by water and gibberellin seed soak. Calif. Agric. 23:18–19.

Chelius, M. K., and Triplett, E. W. 2001. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. :252–263.

Compant, S., Clément, C., and Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42:669–678.

Damalas, C. A., and Koutroubas, S. D. 2018. Current status and recent developments in biopesticide use. Agriculture. 8:13.

García-Fraile, P., Menéndez, E., Celador-Lera, L., Díez-Méndez, A., Jiménez-Gómez, A., Marcos-García, M., et al. 2017. Bacterial probiotics: A truly green revolution. In Probiotics and plant health, Springer, p. 131–162.

Giassi, V., Kiritani, C., and Kupper, K. C. 2016. Bacteria as growth-promoting agents for citrus rootstocks. Microbiol. Res. 190:46–54.

Glick, B. R., Holguin, G., Patten, C. L., and Penrose, D. M. 1999. Biochemical and genetic mechanisms used by plant growth promoting bacteria. World Scientific.

Goswami, D., Thakker, J. N., and Dhandhukia, P. C. 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric. 2:1127500.

Gray, E. J., and Smith, D. L. 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol. Biochem. 37:395–412.

Gutiérrez-Mañero, F. J., Ramos-Solano, B., Probanza, A. n, Mehouachi, J., R. Tadeo, F., and Talon, M. 2001. The plant-growth-promoting rhizobacteria Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins. Physiol. Plant. 111:206–211.

Jia, N., Ding, M.-Z., Luo, H., Gao, F., and Yuan, Y.-J. 2017. Complete genome sequencing and antibiotics biosynthesis pathways analysis of Streptomyces lydicus 103. Sci. Rep. 7:1–8.

Kesavan, P. C., and Swaminathan, M. S. 2018. Modern technologies for sustainable food and nutrition security. Curr. Sci. 115:1876–1883.

Khalid, A., Arshad, M., and Zahir, Z. A. 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J. Appl. Microbiol. 96:473–480.

Lane, D. J. 1. 1991. 16S/23S rRNA sequencing. Nucleic acid Tech. Bact. Syst. :115–175.

Liu, J., Ma, K., Ciais, P., and Polasky, S. 2016. Reducing human nitrogen use for food production. Sci. Rep. 6:1–14.

Van Loon, L. C. 2007. Plant responses to plant growth-promoting rhizobacteria. New Perspect. approaches plant growth-promoting Rhizobacteria Res. :243–254.

Mahmud, K., Makaju, S., Ibrahim, R., and Missaoui, A. 2020. Current progress in nitrogen fixing plants and microbiome research. Plants. 9:97.

Munne-Bosch, S., Onate, M., Oliveira, P. G., and Garcia, Q. S. 2011. Changes in phytohormones and oxidative stress markers in buried seeds of Vellozia alata. Flora-Morphology, Distrib. Funct. Ecol. Plants. 206:704–711.

Orbović, V., Dutt, M., and Grosser, J. W. 2013. Evaluation of the germination potential of citrus seeds during the harvesting season. HortScience. 48:1197–1199.

Ortíz-Castro, R., Contreras-Cornejo, H. A., Macías-Rodríguez, L., and López-Bucio, J. 2009. The role of microbial signals in plant growth and development. Plant Signal. Behav. 4:701–712.

Pingali, P. L. 2012. Green revolution: impacts, limits, and the path ahead. Proc. Natl. Acad. Sci. 109:12302–12308.

Puri, A., Padda, K. P., and Chanway, C. P. 2018. Nitrogen-fixation by endophytic bacteria in agricultural crops: recent advances. Nitrogen Agric. IntechOpen, London, GBR. :73–94.

Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C., et al. 2012. Seed germination and vigor. Annu. Rev. Plant Biol. 63:2012.

Samaras, A., Kamou, N., Tzelepis, G., Karamanoli, K., Menkissoglu-Spiroudi, U., and Karaoglanidis, G. S. 2022. Root Transcriptional and Metabolic Dynamics Induced by the Plant Growth Promoting Rhizobacterium (PGPR) Bacillus subtilis Mbi600 on Cucumber Plants. Plants. 11:1218.

Seldin, L., Van Elsas, J. D., and Penido, E. G. C. 1984. Bacillus azotofixans sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. Int. J. Syst. Evol. Microbiol. 34:451–456.

Soumare, A., Diedhiou, A. G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., et al. 2020. Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. Plants. 9:1011.

Subbiah, V., and Reddy, K. J. 2010. Interactions between ethylene, abscisic acid and cytokinin during germination and seedling establishment in Arabidopsis. J. Biosci. 35:451–458.

Swain, M. R., Naskar, S. K., and Ray, R. C. 2007. Indole-3-acetic acid production and effect on sprouting of yam (Dioscorea rotundata L.) minisetts by Bacillus subtilis isolated from culturable cowdung microflora. Polish J. Microbiol. 56:103.

Taiz, L., Zeiger, E., Møller, I. M., and Murphy, A. 2015. Plant physiology and development. Sinauer Associates Incorporated.

Thokchom, E., Kalita, M. C., and Talukdar, N. C. 2014. Isolation, screening, characterization, and selection of superior rhizobacterial strains as bioinoculants for seedling emergence and growth promotion of Mandarin orange (Citrus reticulata Blanco). Can. J. Microbiol. 60:85–92.

Thomas, B. 2016. Encyclopedia of applied plant sciences. Academic Press.

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A.-C. 2017. Perspectives and challenges of microbial application for crop improvement. Front. Plant Sci. 8:49.

Trivedi, P., Pandey, A., and Palni, L. M. S. 2005. Carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cooler regions. World J. Microbiol. Biotechnol. 21:941–945.

Trivedi, P., Spann, T., and Wang, N. 2011. Isolation and characterization of beneficial bacteria associated with citrus roots in Florida. Microb. Ecol. 62:324–336.

Velten, S., Leventon, J., Jager, N., and Newig, J. 2015. What is sustainable agriculture? A systematic review. Sustainability. 7:7833–7865.

von der Weid, I., Duarte, G. F., van Elsas, J. D., and Seldin, L. 2002. Paenibacillus brasilensis sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil. Int. J. Syst. Evol. Microbiol. 52:2147–2153.

Xi, M., Deyett, E., Ginnan, N., Ashworth, V. E. T. M., Dang, T., Bodaghi, S., et al. 2022. Arbuscular mycorrhizal fungal composition across US citrus orchards, management strategies, and disease severity spectrum. bioRxiv.

Yuan, W. M., and Crawford, D. L. 1995. Characterization of Streptomyces lydicus WYEC108 as a potential biocontrol agent against fungal root and seed rots. Appl. Environ. Microbiol. 61:3119–3128.

Zahir, Z. A., Arshad, M., and Frankenberger, W. T. 2004. Plant growth promoting. Adv. Agron. 81:97.

Zhang, N., Wu, K., He, X., Li, S., Zhang, Z., Shen, B., et al. 2011. A new bioorganic fertilizer can effectively control banana wilt by strong colonization with Bacillus subtilis N11. Plant Soil. 344:87–97

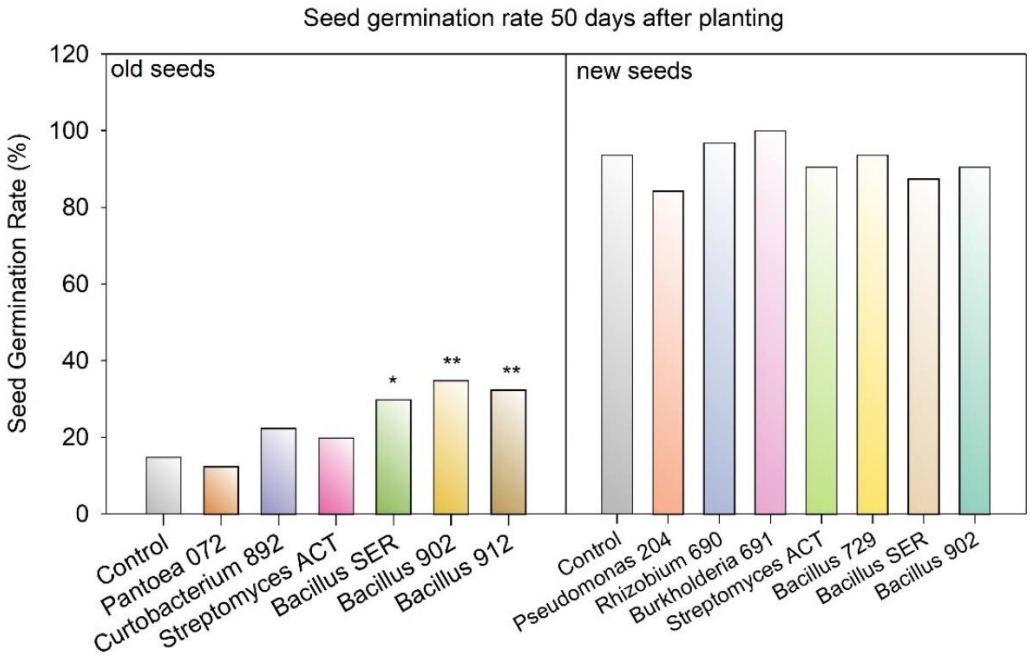

Figure 4.1 Seed germination rate of old and new Carrizo rootstock seed inoculated with bacterial isolates at 50 days after planting. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Chi-squared test).

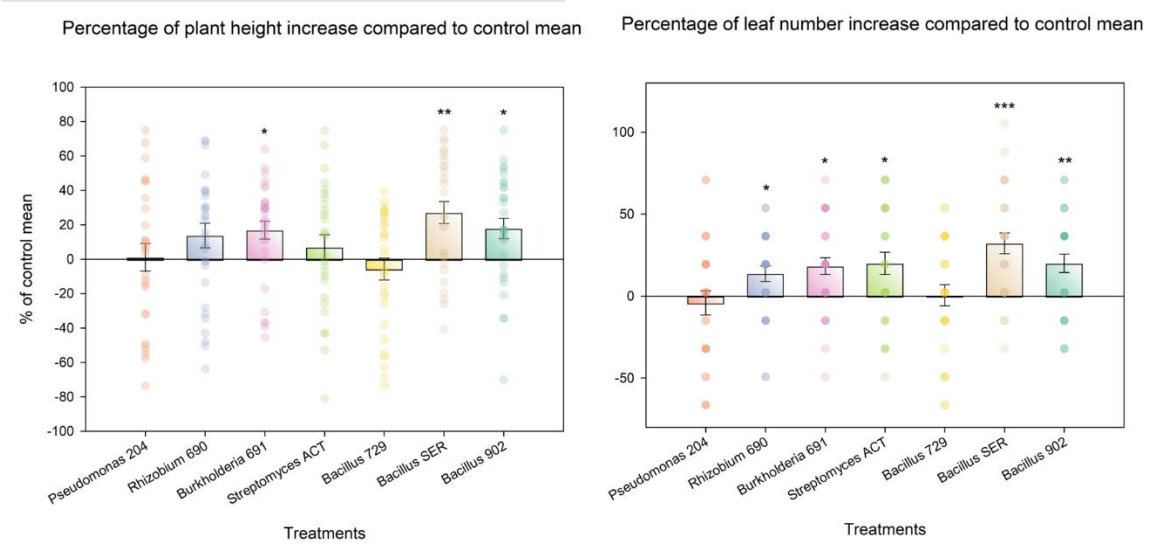

Figure 4.2 Percentage of plant height and leaf number increase compared to control mean of new Carrizo rootstock seedling inoculated with bacterial isolates at 50 days after planting. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student T test).

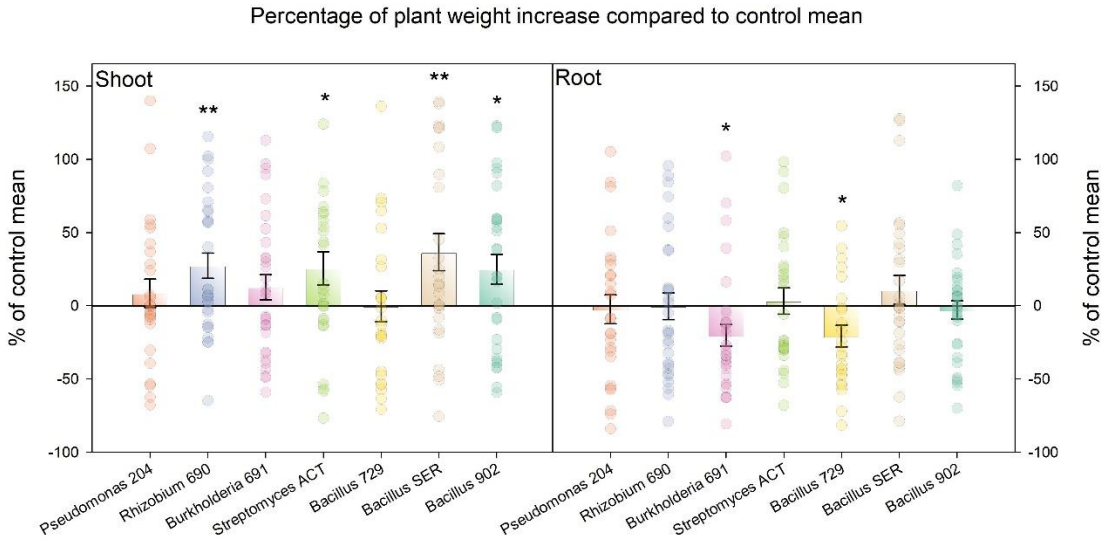

Figure 4.3 Percentage of plant shoot and root fresh weight increase compared to control mean of new Carrizo rootstock seedling inoculated with bacterial isolates at 50 days after planting. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student T test).

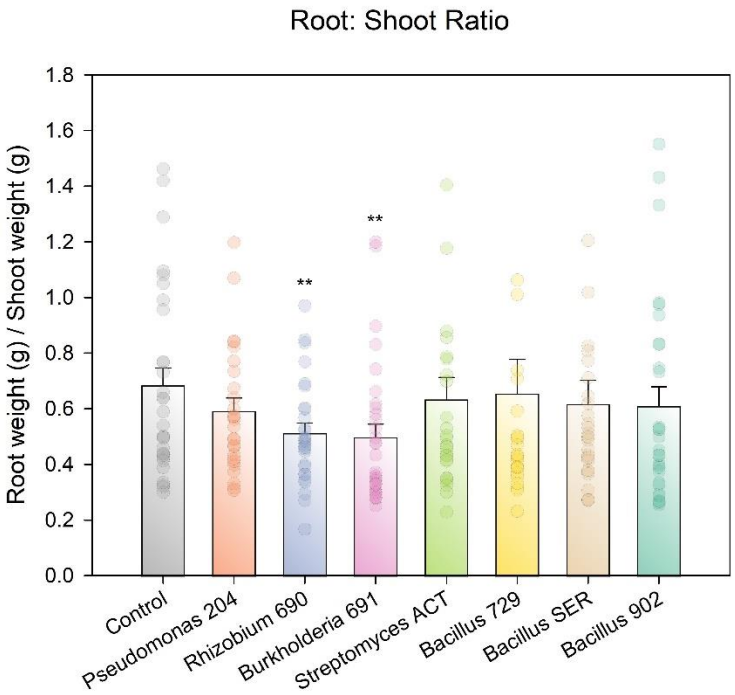

Figure 4.4 Shoot to root ratio of new Carrizo rootstock seedling inoculated with bacterial isolates at 50 days after planting. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student T test).

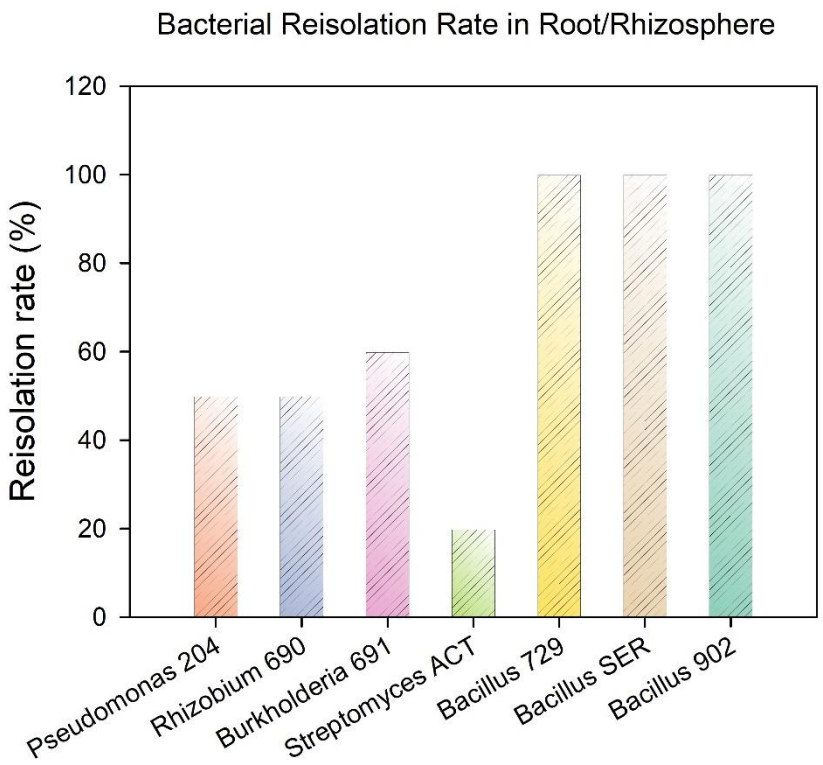

Figure 4.5 Reisolation rate of inoculated bacteria in rhizosphere and root of the new Carrizo rootstock seedling at 50 days after planting.

5 Conclusion

The U.S. citrus industry is valued at $3.33 billion (U.S. Department of Agriculture 2020) and is the 3rd global producer, behind Brazil and China (Jegede 2019). To maintain its competitive position in the global marketplace the US citrus industry needs to address current pressing challenges. The downward trend in both planted acreage and volume of citrus production has been driven by the shrinking Florida industry due to the devastating effects of citrus Huanglongbing disease. Total citrus production has fallen precipitously by 65.3% from its peak in 1998 with orange and grapefruit declines of 71.6% and 80.4%, respectively (Luckstead and Devadoss 2021). Disease management has also driven the cost of production upward in Florida. The total cost of production for processed oranges in 2020-21 was $1,882 per acre, per acre. Foliar sprays of pesticides at $480 per acre represented the largest expense followed by fertilizer at $388 per acre (Singerman 2022). In California, production costs are about $3,500 per acre for oranges with pesticides and fertilizers also accounting for the largest expenses, but costs of water, gasoline and labor are responsible for the production costs differences with Florida.

Fertilizers and pesticides are the foundation of any tree nutritional regime and pests and diseases management program. Nitrogen (N) and phosphorus (P) are the dominant

rate-limiting nutrients in most natural systems and the major constituents of agrochemical fertilizers. California citrus growers applied on average 80-100 lbs N/acre and 40 lbs P/acre to citrus (Geisseler and Horwath 2016). Neonicotinoids have also been extensively used for the control of Asian Citrus Psyllid, the vector of CLas, and the lack of alternative control strategies to manage Huanglongbing suggest that the number of insecticides sprays will increase in years to come and will further increasing production costs. The consequences of neonicotinoids, N and P losses from agricultural land, e.g., through runoff and leaching, can span multiple organizational levels and scales in time and space, and threatens essential ecosystems (Guignard et al. 2017; Sánchez-Bayo et al. 2016; Woodcock et al. 2016).

This study explores the potential of developing integrated citriculture systems that rely on microbial diversity and biological activity as an alternative to chemical-based practices. We provide here a complete profile of the citrus associated bacteriome and mycobiome in several biocompartments (soil, rhizosphere, root endosphere, flower and flush), including a comprehensive description of the root microbial assemblage. Our research established a research pipeline utilizing citrus seeds to evaluate bioinoculants from our biological repository. We identified through metagenomics several additional potential biological targets naturally occurring in citrus that should be further tested as

biofertilizers (Burkholderia, Rhizobium) and biocontrol agents (Epicoccum, Cladosporium). We also showed that the existing commercial products Serenade® with strains of Bacillus act as a growth promoting bacterium in citrus seedlings and could be deployed in nurseries and orchards. Orchard sampling at continental scale in Florida and California coupled with metagenomics approaches indicated that low-input farming practices that foster symbiotic interaction between trees and arbuscular mycorrhizal fungi could lead to extended orchard lifetime because of AMF nutritional benefits and support of defense mechanisms against diseases (Phytophthora root rot, Fusarium dry rot and Huanglongbing). We hope these discoveries will pave the way to a more sustainable and environmentally friendly agriculture.

5.1 Reference

Geisseler, D., and Horwath, W. R. 2016. Citrus Production in California. Available at: https://www.cdfa.ca.gov/is/ffldrs/frep/FertilizationGuidelines/pdf/Citrus_Production_CA.pdf.

Guignard, M. S., Leitch, A. R., Acquisti, C., Eizaguirre, C., Elser, J. J., Hessen, D. O., et al. 2017. Impacts of nitrogen and phosphorus: from genomes to natural ecosystems and agriculture. Front. Ecol. Evol. 5:70.

Jegede, A. 2019. Top 10 Largest Citrus Producing Countries in the World. Available at: http://www.thedailyrecords.com/2018-2019-2020-2021/world-famous-top-10-list/world/largest-citrus-producing-countries-world-statistics-states/6867/.

Luckstead, J., and Devadoss, S. 2021. Trends and Issues Facing the US Citrus Industry. Choices. 36:1–10.

Sánchez-Bayo, F., Goka, K., and Hayasaka, D. 2016. Contamination of the aquatic environment with neonicotinoids and its implication for ecosystems. Front. Environ. Sci. 4:71.

Singerman, A. 2022. Cost of Production for Processed Oranges in Southwest Florida in 2020/21. EDIS. 2022 Available at: https://journals.flvc.org/edis/article/view/129595.

U.S. Department of Agriculture. 2020. Fruit and Tree Nuts Yearbook Tables. Available at: https://www.ers.usda.gov/data-products/fruit-and-tree-nuts-data/fruit-and-tree-nuts-yearbook-tables/.

Woodcock, B. A., Isaac, N. J. B., Bullock, J. M., Roy, D. B., Garthwaite, D. G., Crowe, A., et al. 2016. Impacts of neonicotinoid use on long-term population changes in wild bees in England. Nat. Commun. 7:1–8.