Plant Physiology and Biochemistry 129 (2018) 1–10

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

All roads lead to Rome: Towards understanding different avenues of tolerance to huanglongbing in citrus cultivars

Nabil Killiny[a,*], Shelley E. Jones[a], Yasser Nehela[a], Faraj Hijaz[a], Manjul Dutt[b], Frederick G. Gmitter[b], Jude W. Grosser[b]

[a] Department of Plant Pathology, University of Florida, Citrus Research and Education Center, IFAS, Lake Alfred, FL, 33850, USA
[b] Department of Horticultural Sciences, University of Florida, Citrus Research and Education Center, IFAS, Lake Alfred, FL, 33850, USA

ARTICLE INFO

Keywords:
Citrus
HLB-Tolerance
Metabolites
'Bingo'
Sugar belle
Finger lime
GC-MS
Trimethylsilylation

ABSTRACT

Citrus tolerance to huanglongbing could result from tolerance to the pathogen *Candidatus* Liberibacter asiaticus (*C*Las) and/or to its vector *Diaphorina citri.* Field observations and greenhouse-controlled studies showed that some citrus cultivars were more tolerant than others. However, the mechanism(s) behind the tolerance has not been determined yet. Using GC-MS, we investigated the volatile organic compounds (VOCs) and the non-volatile metabolite profiles of two tolerant citrus cultivars- Australian finger lime, 'LB8-9' Sugar Belle® mandarin hybrid, and a recently released mandarin hybrid 'Bingo'. The three were grafted onto the rootstock, *Carrizo citrange*. Our findings showed that the metabolomic profiles of Australian finger lime were different from that of 'LB8-9'. Finger lime was high in many amino acids and tricarboxylic acid intermediates, whereas 'LB8-9' was high in several amino acids, sugars, and sugar alcohols. 'LB8-9' was high in thymol, which is known for its strong antimicrobial activity against a panel of pathogenic bacteria. The metabolomic profiles of 'Bingo' were intensely different from the other mandarin hybrid, 'LB8-9', including a reduced thymol biosynthetic pathway and low amounts of most of the amino acids and sugar alcohols. Remarkably, 1,8-cineole (eucalyptol) was only detected in 'Bingo', indicating that eucalyptol could have feeding and ovipositional repellency against *D. citri*. The metabolite profiles generated for HLB-tolerant citrus species will improve the ability of citrus breeders and will allow them to take more informed decisions. Metabolomic profiling of HLB-tolerant citrus species could identify tolerance specific markers that can be introduced to other commercial citrus cultivars to improve their tolerance to HLB disease.

1. Introduction

Citrus huanglongbing disease (HLB), also called citrus greening, has decimated many of the world's citrus industries in recent years. In Florida, the disease is caused by *Candidatus* Liberibacter asiaticus, a fastidious gram-negative phloem-limited bacterium transmitted by a tiny Hemipteran insect, the Asian citrus psyllid, *Diaphorina citri* (Garnier et al., 2000; Jagoueix et al., 1996). The bacterium resides and multiplies in the gut and salivary glands of the psyllid vector (Ammar et al., 2011). Psyllids inject the bacteria into host plants through their mouthparts during feeding. Most often, *D. citri* acquires the bacterium as nymphs while feeding on the phloem sap of infected plants (Ammar et al., 2016). After reaching maturity, adults can fly up to 2 km in search of new host plants for feeding and reproduction (Lewis-Rosenblum et al., 2015). Once inoculated into the host tree, the bacteria accumulate in the sieve elements (Achor et al., 2010), consume the supply of nutrients present in the phloem sap (Killiny, 2017), and initiate a cascade of host plant responses which lead to phloem plugging, starch accumulation, and leaf chlorosis (Achor et al., 2010), root loss (Johnson et al., 2014), and reduced yield due to small and lopsided fruit unfit for processing (Gottwald, 2010). Most of citrus cultivars are susceptible to this bacterium and once infected trees show a slow decline in production and often eventually die within 5–10 years (Grafton-Cardwell et al., 2013; Halbert and Manjunath, 2004).

Because the citrus industry has relied heavily on the use of chemical insecticides to combat the insect vector, increasing reports of insecticidal resistance within the Florida psyllid population have appeared (Tiwari et al., 2011). Consequently, citrus growers are in desperate need of alternative strategies such as new, more HLB-tolerant citrus varieties. Because transgenic approaches are not well accepted by consumers, breeding programs remain the best option for the current economic situation. Increasing the genetic diversity through citrus

* Corresponding author. 700 Experiment Station Road, Lake Alfred, Florida, 33850, USA.
E-mail address: nabilkilliny@ufl.edu (N. Killiny).

https://doi.org/10.1016/j.plaphy.2018.05.005
Received 2 March 2018; Received in revised form 19 April 2018; Accepted 2 May 2018

breeding programs has the potential to introduce favorable traits such as disease resistance and reduce the risks associated with monoculture.

Although there are currently no commercial citrus cultivars with strong tolerance to *C*Las, greenhouse studies and field observations showed that some citrus species are more tolerant to *C*Las bacteria than others. Citrus accessions which show some tolerance to HLB have been reported (Albrecht and Bowman, 2012; Cevallos-Cevallos et al., 2012; Folimonova et al., 2009). In a broad study of potentially tolerant hybrids, a 'LB8-9/Sour orange' scion/rootstock combination maintained a healthy appearance and good yield over the five-year study (Stover et al., 2016). Recently, we studied in detail the volatile and non-volatile leaf metabolite profiles of 'LB8-9' Sugar Belle® mandarin hybrid and its closest relatives, 'Dancy' tangerine, 'Minneola' tangelo, 'Duncan' grapefruit, and 'Clementine' mandarin in order to identify compounds related to HLB tolerance (Killiny et al., 2017). In that study, VOCs such as thymol, *p*-cymene, and γ-terpinene were identified as compounds specific to 'LB8-9' which might confer antibacterial properties. In fact, previous studies showed that thymol has a strong antimicrobial activity against a panel of pathogenic bacteria (Du et al., 2015). Phenolic compounds present included benzoic acid, ferulic acid, caffeic acid, and synephrine, and many of these have been implicated in plant response to biotic stresses (Blodgett and Stanosz, 1998; Wallis and Chen, 2012; Wilhelm et al., 2011).

In this follow-up study, we examined the volatile and non-volatile metabolite profiles of 'LB8-9', 'Bingo', and 'Australian Finger Lime' grafted onto 'Carrizo' citrange rootstock. 'Carrizo' citrange is a hybrid of 'Washington' navel orange × trifoliate orange (Castle et al., 2016). "Carrizo' has been in use in Florida since the 1930s (Castle et al., 2016). Its use as a rootstock in Florida is preferred in areas with poorly draining soil because of its tolerance to *Phytophthora* root rot, but it does not perform well under high pH or high salinity conditions (Castle et al., 2016). New evidence suggests that a close citrus relative, 'Australian finger lime' *Microcitrus australasica* (F. Muell.) Swingle showed potential HLB tolerance as *C*Las titers were low for the six years of the study and it was a poor host for *D. citri* (Ramadugu et al., 2016). 'Bingo' mandarin hybrid is a relatively new hybrid consisting of 'Clementine' mandarin × 'Valencia' sweet orange as female parent and 'Seedless Kishu' as male parent (Gmitter, 2015). The original seedling 'Bingo' tree was symptomless and continued to test negative for *C*Las by polymerase chain reaction (PCR) assays through the first 9 years in the field. However, in the 10th year symptoms were observed in leaves and fruit, so the cultivar is apparently not resistant to *C*Las. Its long term tolerance and ability to continue good performance under infection remains to be determined. (Gmitter, 2015). Recently,'Bingo' has become widely available to growers, and several thousand trees have been planted in Florida.

Plants produce a wide variety of complex organic molecules as defenses against bacterial and fungal pathogens (Giamperi et al., 2002; Marchese et al., 2016), animal and insect herbivory (Bennett and Wallsgrove, 1994; Hijaz et al., 2013), and abiotic stresses such as drought or high salinity, and sometimes through interaction of both biotic and abiotic events (Nguyen et al., 2016). Some chemical defense compounds are formed as saps, toxic alkaloids, and phytohormones through their organs (glands, trichomes, leaves, stems, fruit, flowers, etc.), while others may be released into the air to attractant beneficial insects or other pollinators (Bennett and Wallsgrove, 1994). Volatile organic compounds (VOCs) such as terpenes can be easily extracted through use of organic solvents (i.e. hexane, chloroform) while water-soluble metabolites (organic acids, sugars) can be extracted using solvents blended with water. Analysis of these molecules, which are often metabolic pathway intermediates, can help identify subtle changes in complex metabolic systems and can be used comparatively. These studies can be achieved through a variety of analytical tools including liquid and gas chromatography (GC), as well as by using different chemical derivatization techniques. Each method has its own advantages and disadvantages, and may be optimal for some compounds and not others. Using multiple techniques on the same tissue is advantageous in that a more complete picture of the underlying biochemistry can be obtained. Therefore, in this study we chose to extract both the volatile (using hexane extraction) and non-volatile metabolites (using a mixed organic solvent and derivatization) from two aliquots of the same leaf tissue samples. Gas chromatography-mass spectrometry (GC-MS) analysis provided both quantification and identification of compounds from among the four studied cultivars.

In this study, we investigated the volatile and nonvolatile metabolites of finger lime, 'Bingo', 'Sugar Belle' mandarin, and their rootstock 'Carrizo' in order to test if there is a relationship between these metabolites and citrus tolerance to *C*Las and *D. citri*. In our previous study of 'LB8-9' Sugar Belle® mandarin hybrid, tolerance was associated with an overall higher level of extracted VOC compounds than the other cultivars examined (Killiny et al., 2017) as well as the presence of specific antimicrobial compounds. We also expected that the chemical profile of 'Bingo' mandarin hybrid to be closer to 'LB8-9' than finger lime due to its mandarin heritage. Furthermore, by comparing 'Carrizo' citrange rootstock VOC and metabolite profiles to that of the scion/rootstock combinations, specific compounds passed from rootstock to scion may be revealed which may or may not be involved in conferring tolerance to the scion.

2. Material and methods

2.1. Plant material

Healthy one-year old trees of 'Carrizo' citrange trifoliate hybrid [*Citrus sinensis* (L.) Osb. 'Washington' × *Poncirus trifoliata* (L.) Raf.], *Microcitrus australasica* (F. Muell.) Swingle 'Australian finger lime' trees and, two mandarin hybrids, 'LB8-9' Sugar Belle® ['Clementine' mandarin (*Citrus reticulata*) × 'Minneola' tangelo] and 'Bingo' [*Citrus reticulata* hybrid × 'Seedless Kishu' mandarin (*Citrus kinokuni* 'Mukakukishu')] previously grafted onto 'Carrizo' citrange six months earlier were used this study. All trees were maintained together in a climate controlled growth chamber (16 h:8 h light:dark photoperiod, with 27 ± 2 °C and 70 ± 5% RH) prior to sampling. Trees were randomly placed in the greenhouse. Trees were watered three times per week and fertilized monthly with 20:10:20 NPK water soluble fertilizer. Five trees of each type were sampled. Five leaves were harvested from each cultivar on the same day and were frozen at −80 °C overnight before analyses. VOC and metabolites content were extracted from the same biological samples. Samples were analyzed in duplicate [5 replicates x 2 injections] for each type of analysis. Foliar morphology and HLB-tolerance of the cultivars used in this study are presented in Fig. 1 and Table 1. Note the small leaf surface area and the presence of large thorns in finger lime compared to the mandarins, 'LB8-9' and 'Bingo'.

2.2. VOC and metabolite extractions

Leaves were frozen in liquid nitrogen and homogenized using a Tissuelyzer II (Qiagen). Two aliquots of ∼0.1 g leaf tissue were taken from each sample for separate analyses. Leaf volatile organic compounds (VOCs) were extracted from the first tissue aliquot using *n*-hexane exactly as reported in Killiny et al. (2017). Similarly, leaf polar metabolites were extracted from the second aliquot of 0.1 g leaf tissue according to Killiny et al. (2017) except that the extraction solvent was methanol:chloroform:water (8:1:1 v/v/v) and took place at 6 °C overnight on a lab rotator. It should be noted that due to the small leaf size for finger lime and 'Carrizo' trifoliate leaves, whole leaves including the petioles were used for both VOC and metabolite extractions, whereas for larger leaves of 'LB8-9' and 'Bingo' only the middle section of the leaf blade was used for extraction (avoiding the tip and the petiole). All sample sizes were ∼0.1 ± 0.05 g fresh weight.