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Characterization of Young Citrus Rootstock Seedlings Using Metabolomics

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Choice of rootstocks for commercial citrus production is influenced strongly by tolerance to diseases and other stresses. In this study we examined whether rootstocks can be characterized based on their metabolic profile at an early seedling stage and whether presence or absence of specific metabolites can provide clues for understanding specific rootstock traits. We used four commercial citrus rootstock cultivars with different genetic backgrounds: Cleopatra mandarin (*Citrus reticulata*), sour orange (*C. aurantium*), Ridge pineapple sweet orange (*C. sinensis*), and Swingle citrumelo (*C. paradisi* × *Poncirus trifoliata*). Greenhouse-grown seedlings were analyzed for their metabolite composition in leaves and roots via gas chromatography-time of flight-mass spectrometry (GC-TOF-MS). Partial least squares discriminant analysis (PLS-DA) revealed clear metabolic differences among tissue types and rootstock cultivars. In both leaves and roots, largest metabolic differences were observed between 'Swingle' and 'Cleopatra', whereas differences were less pronounced between sour orange and 'Ridge'. Of the 147 chemically identified metabolites, 17 (leaves) and 29 (roots) varied significantly in concentrations among the four rootstocks. Notably, sour orange roots contained highest concentrations of hexitol and myo-inositol, compounds commonly associated with tolerance to osmotic stress, while 'Swingle' roots had lowest concentrations. These observations correspond well with known good and poor field tolerance to unfavorable soil environments for sour orange and 'Swingle', respectively. The information presented in this study provides a foundation for understanding the biochemical mechanisms of rootstock characteristics associated with stress tolerance.

Until the mid-1800s, and well into the 1900s in some regions, citrus was grown predominantly as seedlings (Castle, 2010). Rootstock has been an important component of citrus production in Florida at least since the 1860s and in California since the 1950s (Webber, 1967). Grafting commercial citrus scion varieties on specially selected rootstocks provides the opportunity both to easily propagate trees from mature varieties that will produce fruit many years earlier than an equivalent seedling, as well as to incorporate better tree tolerance to particular diseases and environmental stresses, such as phytophthora root rot, nematodes, high salinity, and cold (Castle, 2010). Of particular importance for citrus crop production is also the effect of rootstock on tree size, yield, and quality of the citrus fruit (Bowman et al., 2016a, 2016b; McCollum and Bowman, 2017). With the increasing awareness of the importance of rootstocks for citrus production, many rootstock cultivars have been developed for their ability to prevent or solve specific agricultural problems or to induce desired horticultural traits.

In this study we examine whether young citrus rootstock seedlings can be characterized by their metabolic profile and if presence or absence of specific metabolites can provide clues for understanding specific rootstock traits. Four commercial citrus rootstock cultivars with different genetic backgrounds and known traits were selected: 'Cleopatra' mandarin (*Citrus reticulata* Blanco), sour orange (*C. aurantium* L.), 'Ridge' pineapple sweet orange (*C. sinensis* L.), and 'Swingle' citrumelo (*C. paradisi* Macf. × *Poncirus trifoliata* L. Raf.). Traits of these and other rootstocks with commercial importance in Florida are described in Castle et al. (2015).

'Cleopatra' is a small-fruited mandarin that has been especially valued as a good rootstock for mandarin-type scions. It is characterized by its tolerance of high salinity, cold hardiness, and resistance of citrus tristeza virus (CTV). Sour orange is a rootstock that has had historical popularity worldwide because it induces good yields and excellent fruit quality, especially when used in combination with grapefruit. Sour orange is characterized by high tolerance to blight, *Phytophthora* foot root, cold hardiness, and adaptability to a wide range of soils. The big drawback of sour orange is its sensitivity to CTV in combination with most scion cultivars, except for lemons (Castle et al., 1993). The incompatibilities of sour orange with most commercial scion cultivars in the presence of CTV accelerated the use of a more diverse range of rootstocks for citrus production, ultimately giving rise to the large number of rootstocks that are hybrids of trifoliate orange (*Poncirus trifoliata*), including 'Swingle' citrumelo.

'Swingle', a hybrid of 'Duncan' grapefruit and trifoliate orange, which was released in 1974 by the U.S. Department of

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Agriculture, has been popular within the Florida citrus industry as a general-use rootstock for many decades because of its high compatibility with most of the commercial scions and its superior performance with grapefruit, sweet orange, and mandarin. It is characterized by high tolerance to cold, resistance to CTV, citrus nematode, and foot rot. Also suitable with most commercial scions such as sweet orange, grapefruit, and mandarin cultivars is the 'Ridge' pineapple, a rootstock that induces vigorous growth and large-sized fruit. Although 'Ridge' shows resistance to burrowing nematode, blight, and CTV, it is susceptible to drought stress, phytophthora root rot and foot rot. Unlike the three rootstocks 'Cleopatra', sour orange, and 'Swingle', 'Ridge' is rarely used as a rootstock, but is nearly identical to other sweet orange cultivars propagated for use as scion (Kesinger, 2015).

Development of new rootstock cultivars involves many years of testing to assess horticultural traits and traits associated with tolerance to stress and diseases. Advanced high-throughput technologies, such as metabolomics, transcriptomics and proteomics, are now available for plant breeders to directly study the relationship between the genotype and the phenotype of a biological system. Among these systems approaches, metabolomics has gained popularity in recent years. Contrary to mRNAs or proteins, metabolites are the end products of cellular regulatory processes and provide direct information on the biological state of a system (Fiehn, 2002). The number of compounds known in the plant kingdom is estimated to be around 200,000, which provides tremendous possibilities for their use in crop breeding programs (Fernie and Schauer, 2008)

Several metabolomic profiling studies have been conducted in citrus, many of them involving postharvest and other citrus fruit-related studies (Jing et al., 2015; Yun et al., 2013). Since the arrival of the devastating bacterial disease Huanglongbing (HLB) in Florida and other citrus producing areas, recent studies have also been directed at deciphering the disease response and the differences of metabolite profiles in susceptible and tolerant citrus varieties (Albrecht et al., 2016; Cevallos-Cevallos et al., 2009, 2012; Chin et al., 2014; Killiny and Hizaj, 2016; Slisz et al., 2012). No information on the basic leaf and root metabolic composition of different citrus rootstock cultivars is available at present. The objective of our study was to examine whether rootstocks can be characterized based on their metabolic profile at an early seedling stage and whether presence or absence of specific metabolites can provide clues for understanding specific rootstock traits. Knowledge of the biochemical composition of different rootstock clones and the associated horticultural traits will aid in accelerating the development of new rootstocks most suitable for citrus production in advance of the unavoidably needed long-term testing under field conditions.

Materials and Methods

Plant material. Plants were grown from seed in the greenhouses of the U.S. Horticultural Research Laboratory in Fort Pierce, FL Seeds were planted into 3.8 × 21 cm Ray-Leach Cone-tainers (Stuewe & Sons, OR) containing a potting medium composed of peat, perlite and vermiculite (Pro Mix BX; Premier Horticulture, Inc., Quakertown, PA). Seedlings were kept under natural light conditions at a temperature of 21 to 28 °C, were irrigated and treated with insecticides as needed, and were fertilized every three weeks using a water-soluble fertilizer mix, 20N–10P–20K (Peters Professional, The Scotts Company, Marysville, OH). Six-month-old seedlings of the four commercial rootstock cultivars Cleopatra mandarin (*Citrus reticulata* Blanco), sour orange (*C. aurantium* L.), Ridge pineapple sweet orange *(C. sinensis* L.*),* and Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata* L. Raf.), were used for metabolite profiling of leaves and roots.

Sample collection. Four to five mature and fully expanded leaves were collected from each plant and immediately frozen in liquid nitrogen. Roots, not exceeding a diameter of 2 mm, were collected, avoiding the pot-bound roots near the bottom of the cone-trainers. Roots were washed under running water to remove potting medium, blotted dry, and frozen in liquid nitrogen. Leaves and roots were collected from six plants of each of the rootstock cultivars. Tissue samples were stored at –80 °C until further use.

Metabolite analysis. Frozen leaves and roots were ground in liquid nitrogen using mortar and pestle. Twenty milligrams of tissue per sample were extracted twice in 1 mL of a mixture of methanol, chloroform and water (5:2:2) for 20 min at 4 °C under constant agitation. After centrifugation at 14,000 × g for 3 min, supernatants were pooled, evaporated to dryness under vacuum in a speedvac concentrator (Savant, Thermo Scientific, Hudson, NH), and stored at –80 °C until GC-TOF-MS analysis.

Metabolite profiling. Methoximation and trimethylsilyation were used as the method of sample derivatization according to Fiehn (Fiehn et al., 2008). Samples were injected into a Gerstel automatic liner exchange system (Gerstel, Muehlheim, Germany) using a Gerstel CIS cold injection system. Gas chromatography and mass spectrometry were performed on an Agilent 6890 gas chromatograph (Agilent, Santa Clara, CA) and a Leco Pegasus IV time of flight mass spectrometer, respectively, both controlled by the Leco ChromaTOF software v.2.32 (Leco, St. Joseph, MI). Data were processed using the algorithms implemented in the open-source BinBase metabolome database as described by Fiehn et al. (2005). Profiling was conducted at the West Coast Metabolomics Center, University of California Davis, CA.

Statistical analysis. Metabolite data were analyzed using the web-based tool MetaboAnalyst v.3.0 including data normalization and statistical analysis (Xia et al., 2015). Supervised partial least squares discriminant analysis (PLS-DA) was used to examine the correlation between data sets and, namely between metabolite concentrations and the sample groupings (rootstock or tissue type). Normalization was conducted by sum, followed by generalized logarithmic transformation. Significant metabolites were extracted from the datasets following analysis of variance (ANOVA) at $P \leq 0.05$ and FDR (false discovery rate) adjustment of 0.1 using tissue type and rootstocks as a factor.

Results and Discussion

Metabolite profiles were established from roots and leaves of 'Cleopatra' (CL), 'Ridge' pineapple (RP), sour orange (SO), and 'Swingle' (SW) seedlings by untargeted GC-TOF-MS. Five hundred unique metabolites were identified, of which 147 (29.4%) were chemically known compounds and 353 (70.6%) were of unknown chemical structure. For the purpose of this study we focused primarily on the group of chemically identified compounds.

Overview of leaf and root metabolites. A total of 318 metabolites were detected that exhibited significantly different concentrations between tissue type and rootstock variety, 96 of which were chemically known.

PLS-DA analysis was used to differentiate between groups of samples defined by tissue type and rootstock cultivar. PLS-DA is a supervised analysis that categorizes samples by using a known

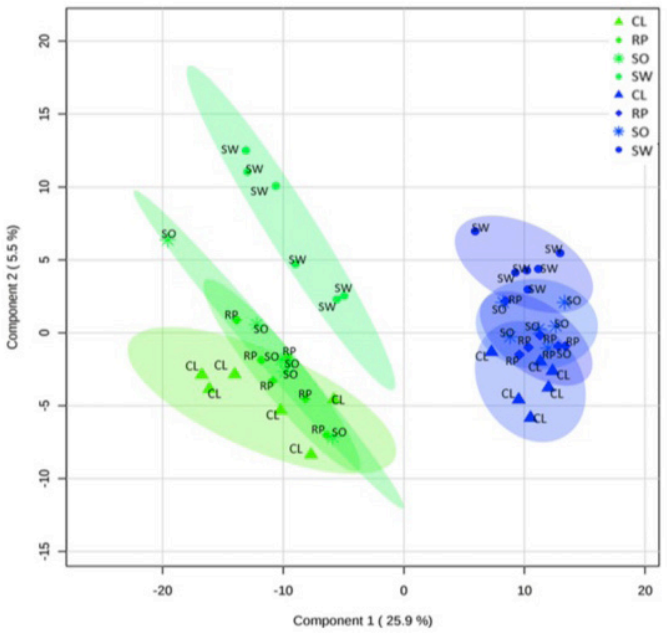

Fig. 1. PLS-DA scores plot of root (blue) and leaf metabolites (green) from four different rootstock seedlings, 'Cleopatra' mandarin (*Citrus reticulata*) (CL), sour orange (*C. aurantium*) (SO), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP), and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW).

classifier, here tissue type and rootstock cultivar. The PLS-DA scores plot showed a clear separation of samples based on tissue type, with 25.9% of the variation explained by component 1 and 5.5% of the variation explained by component 2 (Fig. 1). Tissue type was responsible for most of the separation of samples along component 1, whereas rootstocks separated primarily along component 2. Similar metabolic differences between shoots and roots were observed in other plant species (Gargallo-Garriga, 2014).

Of the known metabolites, 74 were found to be in significantly higher concentration in the leaves compared with roots, independent of the rootstock variety (Table 1). Among these were several metabolites involved in plant defense responses, specifically quinic acid, shikimic acid, dopamine, and putrescine. Quinic acid and shikimic acid are intermediates of the shikimic acid pathway, which provides precursors for the biosynthesis of aromatic amino acids and phenylpropanoids, and other important components of plant signaling and defense pathways (Dewick, 2009). Putrescine belongs to the group of polyamines, phytohormone-like aliphatic amines and known modulators of plant growth and development (Kaur-Sawhney et al., 2003). Besides having antioxidant and anti-senescence properties, polyamines are implicated in the modulation of plant defense responses to diverse environmental stresses, such as drought, salinity and cold (Gill and Tuteja, 2010). Like putrescine, dopamine is also implicated in plant protection against oxidative stress. In addition, it is a member of the catechol family of biochemicals and an important neurotransmitter that functions as a deterrent against herbivores (Kulma and Szopa, 2007; Van Alstyne et al., 2006).

Other metabolites found with significantly higher concentrations in leaves than in roots were those associated with plant primary metabolism, particularly sugars (glucose, fructose, galactose, and raffinose) and many organic acids and TCA cycle intermediates (glyceric acid, malic acid, ferulic acid, saccharic acid, allantoic acid, threonic acid, isothreonic acid, and dehydroascorbic acid). Within the group of unidentified metabolites,

Table 1. Chemically identified metabolites with higher concentrations in leaves than in roots in four commercial citrus rootstock cultivars with different genetic backgrounds: 'Cleopatra' mandarin (*Citrus reticulata*), sour orange (*C. aurantium*), 'Ridge' pineapple sweet orange (*C. sinensis*), and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) independent of the rootstock variety. FDR, false discovery rate. Only metabolites with a fold difference greater than two are shown.

Metabolite	Fold difference	*P*-value	FDR
quinic acid	116.9	< 0.0001	< 0.0001
isothreonic acid	34.5	< 0.0001	< 0.0001
N-acetylmannosamine	28.2	< 0.0001	< 0.0001
putrescine	17.0	< 0.0001	< 0.0001
galactose	16.4	< 0.0001	< 0.0001
dehydroascorbic acid	13.2	< 0.0001	< 0.0001
dihydroxymalonic acid	10.8	< 0.0001	< 0.0001
glyceric acid	10.0	< 0.0001	< 0.0001
raffinose	7.3	< 0.0001	< 0.0001
phytol	7.2	< 0.0001	< 0.0001
phenylalanine	7.1	< 0.0001	< 0.0001
shikimic acid	6.7	< 0.0001	< 0.0001
malic acid	6.3	< 0.0001	< 0.0001
saccharic acid	6.1	< 0.0001	< 0.0001
N-methylglutamic acid	6.1	< 0.0001	< 0.0001
glucose	5.6	< 0.0001	< 0.0001
glycerol-alpha-phosphate	5.2	< 0.0001	< 0.0001
fructose	5.1	< 0.0001	< 0.0001
serine	5.1	< 0.0001	< 0.0001
parabanic acid	5.0	< 0.0001	< 0.0001
threonic acid	4.8	< 0.0001	< 0.0001
1,2-anhydro-myo-inositol	4.3	< 0.0001	< 0.0001
maleic acid	4.3	< 0.0001	< 0.0001
dopamine	4.3	< 0.0001	< 0.0001
galactonic acid	4.3	< 0.0001	< 0.0001
tocopherol alpha	4.2	0.03	0.05
allantoic acid	3.9	< 0.0001	< 0.0001
maleimide	3.8	< 0.0001	< 0.0001
xylonic acid	3.7	< 0.0001	< 0.0001
lactulose	3.7	< 0.0001	< 0.0001
2-ketoglucose dimethylacetal	3.7	< 0.0001	< 0.0001
tyrosine	3.5	< 0.0001	< 0.0001
galactinol	3.4	< 0.0001	< 0.0001
mannonic acid	3.3	< 0.0001	< 0.0001
gluconic acid lactone	3.3	< 0.0001	< 0.0001
ferulic acid	3.0	< 0.0001	< 0.0001
glycerol-3-galactoside	2.9	< 0.0001	< 0.0001
fumaric acid	2.9	< 0.0001	< 0.0001
gluconic acid	2.9	< 0.0001	< 0.0001
mucic acid	2.6	< 0.0001	< 0.0001
N-acetyl-d-hexosamine	2.5	< 0.0001	< 0.0001
N-acetylornithine	2.5	< 0.0001	0.01
beta-mannosylglycerate	2.4	< 0.0001	< 0.0001
ribonic acid	2.4	< 0.0001	< 0.0001
tryptophan	2.4	< 0.0001	< 0.0001
tyramine	2.3	< 0.0001	< 0.0001
pyrrole-2-carboxylic acid	2.3	< 0.0001	< 0.0001
succinic acid	2.2	< 0.0001	< 0.0001
beta-sitosterol	2.2	0.03	0.05
beta-gentiobiose	2.1	< 0.0001	< 0.0001
lysine	2.0	0.02	0.04
lactose	2.0	< 0.0001	< 0.0001

Table 2. Chemically identified metabolites with higher concentrations in roots than in leaves in four commercial citrus rootstock cultivars with different genetic backgrounds: 'Cleopatra' mandarin (*Citrus reticulata*), sour orange (*C. aurantium*), 'Ridge' pineapple sweet orange (*C. sinensis*), and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) independent of the rootstock variety. FDR, false discovery rate.

Metabolite	Fold difference	*P*-value	FDR
glucosaminic acid	13.5	0.002	0.028
hexitol	5.9	< 0.0001	< 0.0001
conduritol-beta-epoxide	4.8	< 0.0001	< 0.0001
cyano-l-alanine	4.3	< 0.0001	< 0.0001
asparagine	3.8	< 0.0001	< 0.0001
lactic acid	3.8	0.0036	0.042
cyanoalanine	3.6	< 0.0001	< 0.0001
2,5-dihydroxypyrazine	3.5	< 0.0001	< 0.0001
scopoletin	2.9	< 0.0001	< 0.0001
pipecolinic acid	2.7	< 0.0001	< 0.0001
ribose	2.7	< 0.0001	< 0.0001
citric acid	2.1	< 0.0001	< 0.0001
levoglucosan	2.0	< 0.0001	< 0.0001

77% were significantly more abundant in leaves compared with roots (data not shown).

Only 13 metabolites with known identity were present in significantly higher concentration in roots than in leaves independent of the rootstock variety (Table 2); these include glucosaminic acid, lactic acid, pipecolinic acid, citric acid, scopoletin, cyanoalanine, cyano-l-alanine and asparagine. Except for glucosaminic acid, none of these compounds was found in more than 6-fold higher concentrations in roots as compared with leaves. Organic acids are components of root exudates and play an important role in the solubilization of otherwise unavailable plant nutrients and in the interaction with beneficial or harmful soil microorganisms (Badri and Vivanco, 2009; Song et al., 2016). Scopoletin is a secondary metabolite with important function in plant defense against abiotic stresses and is also known for its antimicrobial activity (Valle et al., 1997). Similar to our study, higher concentrations of scopoletin were found in roots of the model plant Arabidopsis compared with the aerial parts (Kai et al., 2006).

Cyanoalanine is a byproduct product of the biosynthesis of the stress hormone ethylene and is associated with cyanide metabolism; it is also an intermediate in asparagine biosynthesis (Blumenthal et al.; 1968, Peiser et al., 1984). Cyanogenic compounds are produced in plants as chemical defense against herbivorous insects (Gleadow and Møller, 2014). The higher concentrations of cyanoalanine and asparagine reflect the ability of the roots to not only defend against abiotic but also to biotic stresses. The considerably lower number of known and unknown metabolites present in higher concentrations in roots than in leaves indicates that roots are metabolically less active compared with aboveground counterparts. But, contrary to the known metabolites, for which no more than 13-fold differences were observed, differences for many of the unknown metabolites were considerably (20 to 130-fold) higher (data not shown). Identification of the unknown metabolites will provide new opportunities for discovery of new and biologically important biochemicals.

Leaf metabolites. Seventy-nine leaf metabolites differed significantly in concentrations among the four rootstock cultivars, of which 17 were identified by chemical structure (Table 3). PLS-DA of leaf metabolites resulted in 22.0% of variation along component 1 and 21.9% of variation along component 2 (Fig. 2). Despite considerable sample variation within rootstock variety, the scores plot shows a clear separation of leaf samples from 'Cleopatra' and 'Swingle' whereas little separation was observed between 'Ridge' pineapple and sour orange. The similarity of leaf metabolic profiles observed for 'Ridge' and sour orange is in accordance with their closer taxonomic relationship as both sweet orange ('Ridge' pineapple) and sour orange are thought to have originated from hybridization of pummelo and mandarin which are both considered "true" citrus species (Barrett, 1977). 'Cleopatra' is a mandarin variety and genetically quite different from 'Swingle', which originated from hybridization of grapefruit and trifoliate orange. Although part of the same family (Rutaceae) and subfamily (Aurantioideae), a recent study confirmed that

Table 3. Leaf metabolites with significant differences among rootstock seedlings in four commercial citrus rootstock cultivars with different genetic backgrounds: 'Cleopatra' mandarin (*Citrus reticulata*) (CL), sour orange (*C. aurantium*) (SO), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP) , and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW). FDR = false discovery rate. VIP = variable importance in projection. Metabolites are sorted based on their importance in the PLS-DA model. Averages of non-normalized signal intensities are shown.

Compound	CL	RP	SO	SW	P-value	FDR
shikimic acid	5013	1934	8829	38397	< 0.0001	< 0.0001
dopamine	9976	661	1752	1176	< 0.0001	< 0.0001
myo-inositol	15301	8221	22525	169841	< 0.0001	< 0.0001
glycerol-3-galactoside	2642	1499	3771	7804	0.01	0.05
beta-mannosylglycerate	1455	797	1582	4554	< 0.0001	0.04
sorbitol	2362	1167	2232	7034	0.01	0.05
6-deoxyglucose	1252	650	938	4397	< 0.0001	< 0.0001
citric acid	47024	17051	28899	240917	< 0.0001	< 0.0001
mucic acid	1194	351	1463	680	0.01	0.09
isothreonic acid	28570	2175	14973	13767	< 0.0001	< 0.0001
succinic acid	1217	911	1005	1511	0.01	0.05
conduritol-beta-epoxide	103134	23038	30117	476091	< 0.0001	0.01
pipecolinic acid	868	207	411	1676	0.01	0.08
threonic acid	2863	1691	4919	4837	0.01	0.08
2-ketoglucose dimethylacetal	691	536	938	943	0.01	0.09
hexaric acid	218	153	664	278	0.01	0.05
urea	26618	3166	6103	47551	< 0.0001	0.03

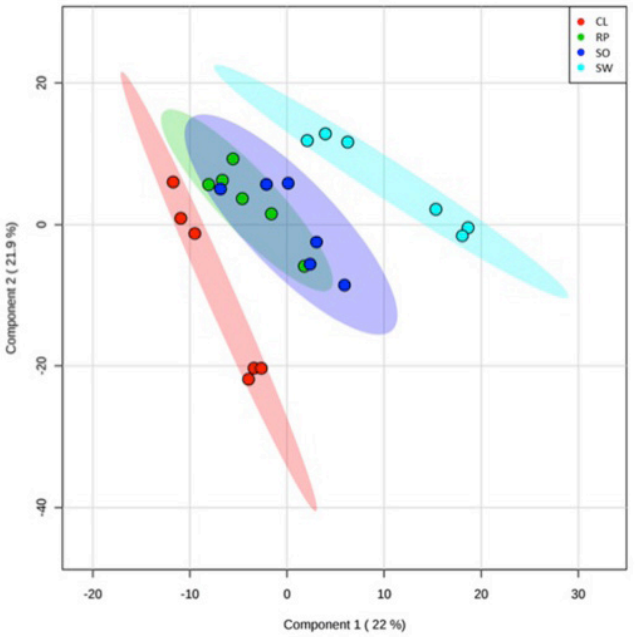

Fig. 2. PLS-DA scores plot of leaf metabolites from four different rootstock seedlings, 'Cleopatra' mandarin (*Citrus reticulata*) (CL, red), sour orange (*C. aurantium*) (SO, blue), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP, green) , and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW, turquoise) .

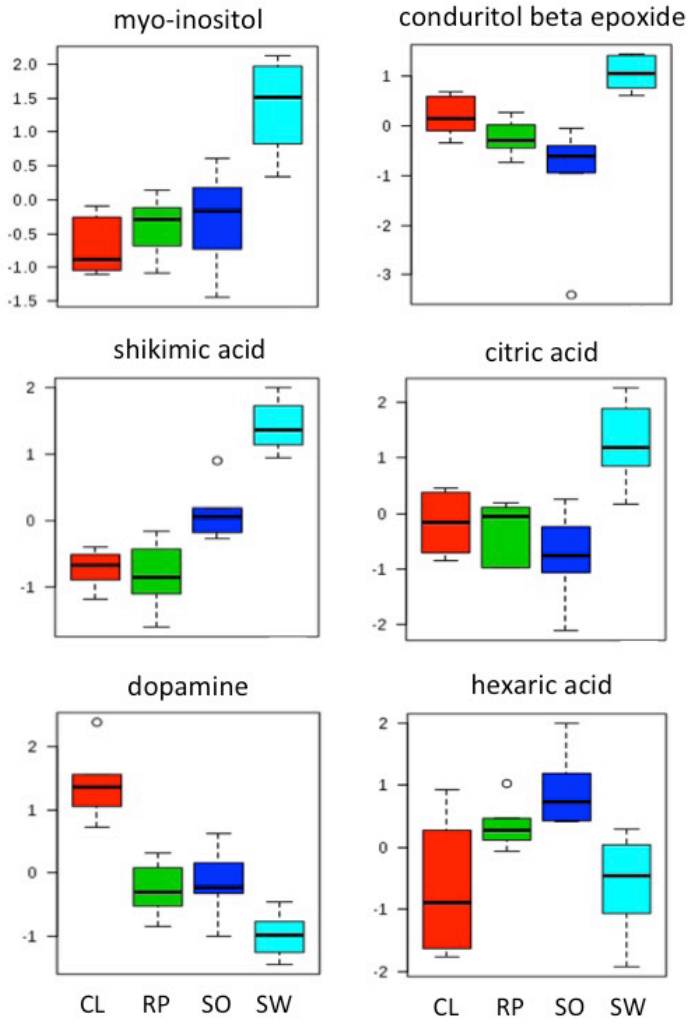

Fig. 3. Boxplots of selected leaf metabolites with significant differences among rootstock cultivars, 'Cleopatra' mandarin (*Citrus reticulata*) (CL, red), sour orange (*C. aurantium*) (SO, blue), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP, green) , and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW, turquoise) . Box length represents the interquartile range, and the black center line indicates the median for each dataset. Outliners are marked as circles.

Poncirus forms a genus that is separate from *Citrus* (Wu et al., 2018). This demonstrates that the metabolic composition clearly is associated with the taxonomic relationship of the organisms investigated.

Many leaf metabolites of unknown chemical identity were found in highest concentrations in 'Swingle' compared with the other three rootstock cultivars (data not shown), further reflecting the greater genetic distance of 'Swingle'. Among the known compounds with highest concentrations in 'Swingle' were myo-inositol, conduritol-beta-epoxide, shikimic acid, and citric acid (Fig. 3). Other metabolites were found in highest concentrations in leaves from other rootstocks, and include dopamine, which was most abundant in 'Cleopatra' leaves and hexaric acid, which was most abundant in sour orange leaves.

Root metabolites. Although considerably fewer metabolites were more abundant in roots compared with leaves, more (101) differed significantly among the four rootstock cultivars. Of the 101 significantly different metabolites, 29 were chemically identified (Table 4). PLS-DA revealed 35% of variation along component 1 and 9.6% of variation along component 2 (Fig. 4). Similar to leaves, 'Cleopatra' and 'Swingle' root samples separated most strongly. Compared with the leaves, root samples from 'Ridge' pineapple and sour orange separated more clearly, although some overlap between samples was observed. This confirms that metabolic profiles correspond well with taxonomic relationships. Several of the known metabolites were present in highest concentrations in sour orange roots, particularly hexitol, myo-inositol, and 1,2-anhydro-myo-inositol. Sugar alcohols such as hexitol and myo-inositol play important roles in the tolerance of plants to salt and osmotic stress (Kusvuran et al., 2013; Sengupta et al. 2015). The finding of higher levels of these compounds in roots of sour orange corresponds with the known field performance of sour orange which adapts well to many unfavorable soil environments. Interestingly, of the four rootstocks, 'Swingle' harbored lowest concentrations of hexitol, myo-inositol, and 1,2 anhydromyoinositol in the roots, which is the opposite of what was observed in the leaves. In comparison with sour orange and 'Cleopatra',' Swingle' is more sensitive to unfavorable abiotic soil conditions; this suggests the possibility of using polyols as metabolic markers for rootstock tolerance of difficult soils.

Like myo-inositol and the other sugar alcohols, conduritol-beta-epoxide (CBE) was found in lowest concentrations in 'Swingle' (Fig. 5). CBE is the racemate of D- and L- 1,2 anhydro myo-inositol and an important inhibitor of acid beta-glucosidases and alpha glucosidases, important enzymes involved in the chemical defense of plants against pathogens and herbivores (Morant et al., 2016). Among the reasons why trifoliate hybrid rootstocks such as 'Swingle' have become popular is their tolerance to citrus tristeza virus and to specific soilborne diseases. It is possible that the low concentrations of CBE and consequently, higher activity of chemical defense enzymes may play a part in these rootstock traits. In general, trifoliate-type rootstocks also show higher tolerance to HLB, although the degree varies among varieties (Albrecht and Bowman, 2012; Ramadugu et al., 2016).

Table 4. Root metabolites with significant differences among four citrus rootstock seedlings in four commercial citrus rootstock cultivars with different genetic backgrounds: 'Cleopatra' mandarin (*Citrus reticulata*) (CL), sour orange (*C. aurantium*) (SO), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP), and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW). FDR = false discovery rate. VIP = variable importance in projection. Metabolites are sorted based on their importance in the PLS-DA model. Averages of non-normalized signal intensities are shown.

Compound	CL	RP	SO	SW	P-value	FDR
phosphate	10768	5339	4671	1576	<0.0001	<0.0001
threonic acid	337	279	688	380	<0.0001	0.01
fucose	159	151	202	178	<0.0001	<0.0001
fumaric acid	637	642	997	912	<0.0001	0.03
conduritol-beta-epoxide	684796	286014	356319	117927	0.01	0.06
maleic acid	391	346	529	417	<0.0001	0.04
hexuronic acid	243	180	305	205	0.01	0.05
guanidinosuccinate	212	181	273	196	0.01	0.06
xylose	761	820	1908	757	0.01	0.05
putrescine	4047	2802	3604	3350	<0.0001	0.02
squalene	476	718	751	478	<0.0001	0.02
maltitol	192	173	183	179	0.01	0.06
gluconic acid	378	118	147	87	<0.0001	0.02
galactonic acid	1022	176	557	133	<0.0001	0.01
parabanic acid	734	667	719	618	0.02	0.09
mannonic acid	594	132	374	101	<0.0001	0.01
sucrose	374317	357324	460276	223060	0.01	0.07
2,5-dihydroxypyrazine	781	450	515	674	<0.0001	<0.0001
myo-inositol	38005	23141	53286	6205	0.01	0.06
saccharic acid	1058	271	835	227	<0.0001	<0.0001
hexitol	166590	99253	473430	19571	<0.0001	<0.0001
trans-4-hydroxyproline	4170	349	636	739	<0.0001	0.04
hexaric acid	111	167	197	91	0.01	0.07
phytol	3291	887	2460	1620	<0.0001	0.02
scopoletin	468	201	220	320	0.01	0.08
tryptophan	4916	1261	2846	2244	<0.0001	0.02
lyxitol	224	577	199	456	0.02	0.09
alpha-ketoglutarate	741	760	858	299	0.01	0.06
1,2-anhydro-myo-inositol	443	395	606	154	0.02	0.09

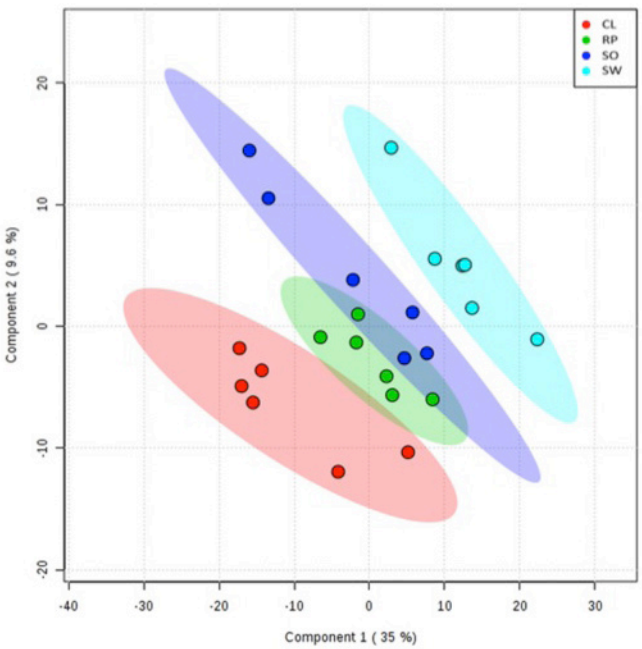

Fig. 4. PLS-DA scores plot of root metabolites from four different rootstock seedlings, 'Cleopatra' mandarin (*Citrus reticulata*) (CL, red), sour orange (*C. aurantium*) (SO, blue), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP, green), and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW, turquoise).

Several metabolites were found in highest concentrations in 'Cleopatra' roots compared with the other rootstocks, particularly trans-4-hydroxyproline, conduritol-beta-epoxide, galactonic acid, and saccharic acid. Like sour orange, 'Cleopatra' is a rootstock with good adaptability to difficult soils and stands out particularly in its ability to tolerate high salinity conditions (Zekri and Parsons, 1989). Trans-4-hydroxyproline, is a non-proteinogenic amino acid; its precursor proline is known to accumulate in plants in response to many different biotic and abiotic stresses. Like the sugar alcohols, proline is considered an osmoprotectant or compatible solute. In many plant species, high concentrations of proline were correlated with tolerance to salt stress (Petrusa et al., 1997; Hayat et al., 2012). Interestingly, hydroxyproline is an important component of the plant cell wall, forming the large group of hydroxyproline-rich glycoproteins (HRGPs) which play important roles in plant development and have also been implicated in the response of plants to salt stress (Zagorchev et al., 2014). Whether trans-4-hydroxyproline has a causative association with the good field performance of 'Cleopatra' in conditions of high salinity remains to be investigated.

Like trans-4-hyroxy proline, the monosaccharide fucose is a structural component of plant cell walls; it is also one of the major components of mucilage secreted by plants roots (Roy et al., 2002). Besides enhancing the quality of the soil, root mucilage plays an important role in the interaction of roots with the soil microbial community. Of the four rootstocks, fucose was detected in highest

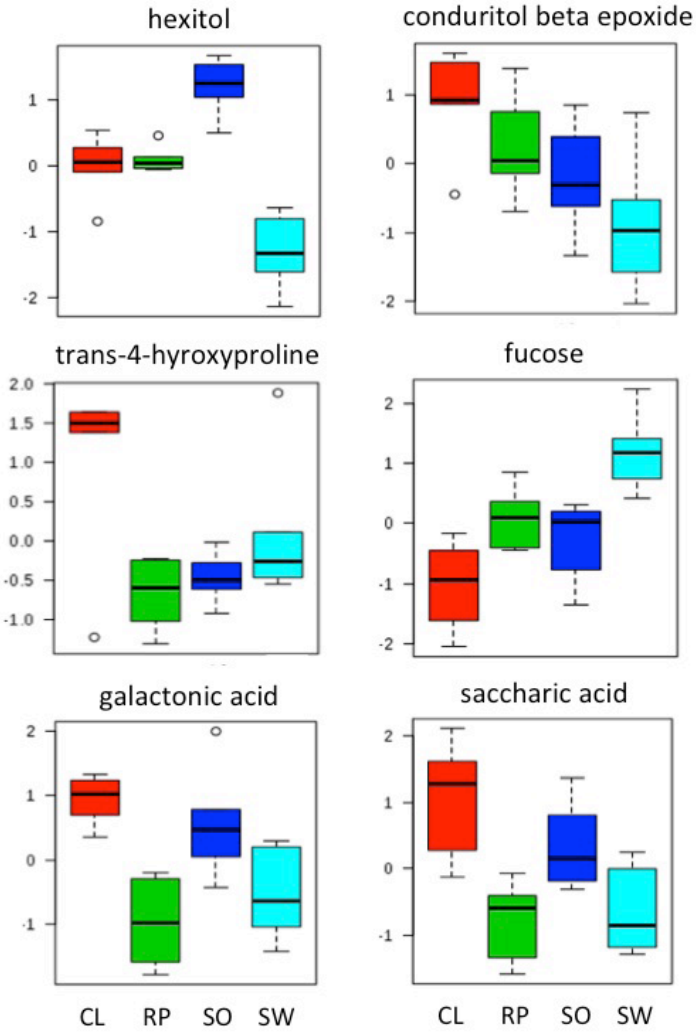

Fig. 5. Boxplots of selected root metabolites with significant differences among rootstock cultivars, 'Cleopatra' mandarin (*Citrus reticulata*) (CL, red), sour orange (*C. aurantium*) (SO, blue), 'Ridge' pineapple sweet orange (*C. sinensis*) (RP, green), and 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) (SW, turquoise). Box length represents the interquartile range, and the black center line indicates the median for each dataset. Outliners are marked as circles.

concentrations in 'Swingle' roots and in lowest concentrations in 'Cleopatra' roots. The large quantities of fucose present in the root mucilage of maize were suggested to have significance for the recognition of maize roots by parasitic root fungi (Northcote and Gould, 1989). Different concentrations of fucose may also play a role in the different interaction of citrus rootstocks with soil-borne pathogens.

The sugar acids saccharic acid (syn. glucaric acid) and galactonic acid show a similar profile, with highest concentrations in 'Cleopatra' roots and lowest concentration in 'Ridge' pineapple and 'Swingle' roots. The exact role of these metabolites in the response of plants to different biotic and abiotic influences is unclear, but galactonic acid, a precursor of ascorbic acid, was implicated in the response of Arabidopsis plants to high salt conditions (Kempa et al., 2008).

Conclusions

Many metabolites were detected in leaves and roots of young greenhouse grown citrus rootstock seedlings. The metabolic profiles of both leaves and roots corresponded well with the taxonomic relationships of the rootstocks used in this study but were more clearly defined for the roots. Although many more metabolites were present in higher concentrations in the leaves than in roots, more root metabolites were found to differ significantly among rootstock varieties. Several metabolites were identified that may be associated with rootstock traits, such as hexitol and myo-inositol, and their relative concentrations in the roots correspond with field tolerance of rootstock cultivars to unfavorable soil environments. Most of the metabolites with significant differences among rootstock genotype were of unknown chemical identity but were valuable for establishing taxonomic relationships. They also present a resource for future discovery of new biologically important molecules. The results from this study provide a foundation for further studies to decipher the biochemical composition of rootstocks and their possible relationships with rootstock-specific traits.

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