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NMR Analysis Reveals a Wealth of Metabolites in Root-Knot Nematode Resistant Roots of *Citrullus amarus* Watermelon Plants

Mihail Kantor,[1,2] Amnon Levi,[2] Judith Thies,[2] Nihat Guner,[3] Camelia Kantor,[4] Stuart Parnham,[5] and Arezue Boroujerdi[1*]

[1]Claflin University, 898 Goff Ave., Orangeburg, SC 29115.

[2]USDA, ARS, U.S. Vegetable Laboratory, 2700 Savannah Highway, Charleston, SC 29414.

[3]Sakata Seed America, 18095 Serene Drive Morgan Hill, CA 95037.

[4]USGIF, 2325 Dulles Corner Blvd, Suite 450, Herndon, VA 20171.

[5]Marine Biochemical Sciences Hollings Marine Laboratory National Institute of Standards and Technology, 331 Fort Johnson Road, Charleston, SC, 29412.

*E-mail: abouroujerdi@claflin.edu.

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Abstract

Citrullus amarus (*CA*) (previously known as *Citrullus lanatus* var. *citroides*) accessions collected in southern Africa are known to have resistance to root-knot nematodes (RKN) and are suitable rootstocks for grafted watermelon. The objective of this study was to conduct a comparative metabolomics analysis and identify unique metabolites in roots of *CA* accessions versus roots of watermelon cultivars (*Citrullus lanatus* (Thunb.) Matsum. and Nakai var. *lanatus; CL*). Nuclear magnetic resonance (NMR) technology and principal component analysis (PCA) were used to analyze and compare metabolic profiles of seven *CA* accessions resistant to RKN along with two RKN-susceptible watermelon cultivars (Charleston Gray and Crimson Sweet). Calculation of the Mahalanobis distance revealed that the *CA* United States Plant Introduction (PI) 189225 (Line number 1832) and PI 482324 (1849) have the most distinct metabolic profiles compared with the watermelon cultivars Charleston Gray and Crimson Sweet, respectively. Several amino acids identified in the *CA* accessions were reported in previous studies to have a nematicidal effect. The results in this study indicate that roots of watermelon accessions collected in the wild are rich in metabolic compounds. These metabolic compounds may have been diminished in watermelon cultivars as a consequence of many years of cultivation and selection for desirable fruit qualities.

Key words

Biochemical pathway analysis, *Citrullus lanatus*, *Citrullus amarus*, Nuclear magnetic resonance, Root-knot nematodes, Watermelon roots.

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai var. *lanatus*) (*CL*) is among the most commonly cultivated vegetable crops worldwide. Africa is the center of origin (Jarret et al., 1997; Levi et al., 2001, 2011; Chomicki and Renner, 2015) with several *Citrullus* spp. (Chomicki and Renner, 2015; Paris 2015) exhibiting wide genotypic and phenotypic diversity (Levi et al., 2013, 2016). *CL*, which includes the sweet dessert watermelon, exists in central and north Africa, also includes a closely related type used for seed consumption known as "Egusi" watermelon (Dahl Jensen et al., 2011). Although watermelon cultivars have close genetic relationships with Egusi watermelons (Guo et al., 2013; Levi et al., 2013, 2016), the later type is also classified as "*Citrullus mucosospermus*" (Chomicki and Renner, 2015). Levi et al. (2001) indicated that watermelon cultivars share a narrow genetic base. Consequently, they are highly susceptible to soil-borne diseases and pests compared with their counterpart *Citrullus* accessions collected in the wild (Thies and Levi, 2003; Thies and Levi, 2007; Thies et al., 2012, 2015a; Wechter et al., 2012a, 2012b; Levi et al., 2013).

Until recent years, preplant fumigation of soil beds with methyl bromide has been the main method for suppression of RKN in watermelon fields. Indeed, about 6% of methyl bromide applied for preplant soil fumigation in vegetable crops worldwide was used for watermelon and melon (USDA, 1993). However,

methyl bromide has been excluded from the market (U.S. Environmental Protection Agency, 2012) and there is a continuous need to identify and develop new alternatives to combat RKN from attacking vegetable crops. Identifying new resistance sources and utilizing them in breeding for resistant or tolerant varieties is considered the most effective approach for controlling RKN (Mercer and Watson, 1996). Plant resistance could reduce RKN infestation through abiosis, while tolerance allows the plant to sustain RKN presence. A watermelon type that exists in southern Africa and named by native people as cow watermelon, *Citrullus amarus* (*CA*), and until recently known in the USA as the Citron watermelon or *Citrullus lanatus* var. *citroides* (Chomicki and Renner, 2015), represents a wide genetic diversity and has a viable gene pool useful for improvement of watermelon cultivars for disease or pest resistance (Levi et al., 2013). United States Plant Introductions (PIs) of *CA,* collected in southern Africa, were identified as resistant to root-knot nematodes (RKN). These *CA* PIs showed lower gall formation and low RKN egg masses compared with watermelon cultivars (Thies and Levi, 2003; Thies and Levi, 2007). Experiments in fields infested with RKN in the southeast United States showed that *CA* accessions are robust rootstocks for seedless watermelon while other commercial rootstock cultivars representing *Cucurbita* spp. or *Lagenaria siceraria* succumbed to RKN (Thies et al., 2010, 2012, 2015a, 2015b, 2015c; Levi et al., 2014). These studies indicate that the *CA* accessions have natural resistance against RKN which does not exist in watermelon cultivars (*CL*), or in pumpkin, squash, or bottle gourd rootstocks used for grafted watermelon.

Plants have a wide range of defense mechanisms by which they respond to biotic and abiotic stresses, mainly in producing defense metabolites. Plant secondary metabolites are an integral part of the host plant's resistance mechanisms against disease causing organisms, or for interaction with beneficial organisms (Wink, 2003). Prior research using nuclear magnetic resonance (NMR) technology has focused on analyzing watermelon fruits, seeds and leaves (de Miranda Costa et al., 2007; Mahmud et al., 2015) or on finding phytochemicals in the desert watermelon *Citrullus colocynthis* that have an important medicinal value (Chawech et al., 2015). Although there is sufficient information on roots of RKN-resistant *CA* accessions versus watermelon cultivars (*CL*) (Thies and Levi, 2003, 2007), there has not been any study comparing their root metabolites. The overall objective of this study was to elucidate the root metabolite profiles of RKN-resistant *CA* accessions versus watermelon cultivars using NMR technology.

Materials and methods

Plant material

Seeds of seven homozygous resistant germplasm lines, each derived (through self-pollination and single seed descent through four-five generations) from a PI resistant to RKN (*CA*) and watermelon cultivars (*CL*; Crimson Sweet and Charleston Gray) susceptible to RKN (Thies and Levi, 2003, 2007) were grown in a greenhouse at the USDA, ARS, U.S. Vegetable Laboratory in Charleston, SC. All seven PIs showed similar levels of resistance in the field studies performed by Thies and Levi (2007). The seeds were sowed in trays with individual cells containing a steam-sterilized Metro Mix 360 Potting Soil (Sungro, Agawam, MA) free of nematodes (Table 1). On the third day after emergence, and still in the absence of nematodes, seedlings were collected for root analysis.

Preparation of plant materials for NMR analysis

On the third day after emergence, the seedlings not exposed to nematode infection were removed from soil and roots were washed several times with distilled cold water to remove soil debris. Then, roots were blotted in a paper towel to remove water, and then dipped in liquid nitrogen to quench all metabolic processes and prevent any stress response from sample processing. The frozen roots were then lyophilized for 48 hr, at −40°C, and 0.05 MPa, to remove all water and permanently quench metabolism. Once the samples were dried, any remaining soil debris was removed from the roots using forceps. All roots were collected from each plant and their dry weight was recorded. Roots of six plants with similar size (fresh and dry weight) were selected to represent each of the nine genotypes and each of these six root samples was used separately for metabolite extraction and analysis.

Metabolite extraction

The water content of the roots was calculated using the dry mass and used in determination of the methanol, chloroform, and water ratio (2:2:1.8). Methanol–chloroform–water extractions of the dried rootstocks were performed as described by Kim et al. (2010). Between 3 and 5 mg of dried roots were grinded using mortar and pestle. The exact masses of each replicate were recorded. The polar solvents were added in the mortar in order to recover as much plant materials as possible. The solution was then trans-

Table 1. Germplasm lines of *Citrullus amarus* (*CA*) and watermelon cultivars (*Citrullus lanatus; CL*) used in this study.

Watermelon germplasm line/cultivar	Parental PI of germplasm line	*Citrullus* spp.
W2001	PI 482303	*CA*
W1832	PI 189225	*CA*
W1849	PI 482324	*CA*
W1254	PI 244017	*CA*
W1446	PI 244018	*CA*
W1813	PI 482319	*CA*
W1482	PI 482259	*CA*
Charleston Gray		*CL*
Crimson Sweet		*CL*

ferred to a glass conical vial containing chloroform and water. After vortex-mixing, the samples were kept on ice for 10 min. The samples were centrifuged at 4°C at 2,000×*g* for 10 min. The polar and nonpolar phases of the sample were separated, and the polar solvents of each sample were removed using a Vacufuge Plus concentrator (Eppendorf AG, Hamburg, Germany).

NMR sample preparation and spectroscopy

Sample preparation and NMR spectroscopy followed the procedure described by Mahmud et al. (2015). After the solvents were completely removed from the polar fraction, 620 µL of NMR buffer (100 mM sodium phosphate buffer (pH 7.3), 1 mM 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (internal standard, TMSP; CAS Registry No. 24493-21-8), and 0.1% sodium azide, in 99.9 atom % D_2O) were added to each sample. The samples were then vortex-mixed and centrifuged to remove any remaining undissolved material. From each sample, 600 µL were transferred into 5 mm NMR tubes (Norell). The NMR spectra were recorded on a Bruker Avance III spectrometer (Bruker Corporation, Billerca, MA, USA) operating at 700 MHz. The first increment of a presat-noesy experiment was acquired using the standard Bruker noesypr1d pulse sequence. This resulted in a one-dimensional ^1H spectrum (metabolic profile) for each sample (9 genotypes × 6 replicates). All data were collected using a spectral width of 16.0 ppm and 64K points resulting in an acquisition time of 2.9 sec. The spectra were collected with 120 scans, 4 dummy scans, 3 sec relaxation delay, and pre-saturation at the residual water frequency. The 90° pulse widths were measured for each sample using the automatic pulse calculation experiment (pulsecal) in TopSpin 2.1.1 (Bruker Bio Spin, Billerica, MA, USA). Two-dimensional ^1H–^{13}C heteronuclear single quantum correlation (HSQC) data were collected for a representative *CA* sample and one for each cultivar using the Bruker hsqcedetgpsisp2.2 pulse sequence. The ^1H was observed in the F2 channel with a spectral width of 11 ppm, whereas the ^{13}C was observed in the F1 channel with a spectral width of 180 ppm.

Data analysis

After the ^1H NMR data was collected on each of the 54 individual samples in this study, the first step was to generate bucket tables using AMIX software (version 3.9.7, BrukerBioSpin). Once data were normalized to total intensities, spectra were binned into 0.01 ppm wide buckets over the region of 0.5–10.0 ppm using advanced bucketing in AMIX. Two exclusions were made, one for the water region (4.75–4.90 ppm) and one for methanol contamination (3.39–3.27 ppm). Next, principal component analysis (PCA) was performed on the bucket tables generated from AMIX using MetaboAnalyst3.0 (Xia and Wishart, 2016). The Mahalanobis distance (D_M), two-sample Hotelling's *T*2 statistic (*T*2), *F* values (*F*t) and critical *F* values (*F*c) were calculated using MatLabR2010b.31 for each score plot generated during the analysis (Goodpaster and Kennedy, 2011).

The next step was to identify the metabolites that were significantly different in concentration when the resistant *CA* and susceptible *CL* lines were compared. MetaboAnalyst3.0 was used to determine whether or not the changes in bucket intensities were statistically significant. Buckets with corresponding *p* values <0.05 were considered statistically significant. Lastly, the metabolites corresponding to the peaks in the buckets were identified using the Biological Magnetic Resonance Data Bank (BMRB) (www.bmrb.wisc.edu/metabolomics/), Madison Metabolomics Consortium Database (MMCD) (http://mmcd.nmrfam.wisc.edu/), ChenomX NMR Suite (ChenomX Inc., Edmonton, AB, Canada), and published studies (Mahmud et al., 2014). Metabolic data interpretation was facilitated with the Metabolic Pathway Analysis (MetPA) component of MetaboAnalyst3.0 (Xia and Wishart, 2016).

Results

The first step in this study was to compare the metabolic profiles of roots of 3 day old seedlings of *CA* PIs versus those of the watermelon cultivars Charleston Gray

Table 2. List of metabolites identified in *Citrullus amarus* or *Citrullus lanatus* by using 1D ^{1}H and 2D ^{1}H–^{13}C-NMR spectroscopy. All chemical shifts for H–C pairs in each metabolite are given with bold chemical shifts corresponding to the peaks labeled in the 1D ^{1}H noesy and 2D ^{1}H–^{13}C HSQC spectra. Un-bold chemical shift estimations are given for H–C pairs that were not identified in the spectra due to overlap with other peaks or noise.

No.	Metabolite	^{1}H chemical shifts (ppm) (functional group or specific H, multiplicity of peak)	^{13}C chemical shifts (ppm) (functional group or specific C)
1	4-Aminobutyrate	**1.92** ($^{2}CH_2$); **2.30** ($^{1}CH_2$); **3.02** ($^{3}CH_2$)	**26.6** ($^{2}CH_2$); **37.2** ($^{1}CH_2$);**42.0** ($^{3}CH_2$)
2	2-Oxoglutarate	**2.50** ($^{1}CH_2$, t); **3.02** ($^{2}CH_2$, t)	33 ($^{1}CH_2$); 39 ($^{2}CH_2$)
3	Acetate	**1.92** (CH_3, s)	26 (CH_3)
4	Alanine	**1.48** (H^{β},d); 3.8 (H^{α},q)	**19.0** (C^{β}); 53 (C^{α})
5	Arginine	**3.25** (H^{δ},t); **1.66**, **1.74** (H^{γ},m); **1.92** (H^{β},m); 3.8 (H^{α},t)	43 (C^{δ}); 27 (C^{γ}); 30 (C^{β}); 57 (C^{α})
6	Asparagine	**2.87, 2.96** (H^{β},dd); 4.0 (H^{α},q)	**37.5** (C^{β}); 54 (C^{α})
7	Aspartate	**2.70**, **2.82** (H^{β},dd); **3.90** (H^{α},q)	**39.4** (C^{β}); **55.1** (C^{α})
8	Betaine	**3.27** (CH_3, s); **3.91** (CH_2, s)	56 (CH_3); 69 (CH_2)
9	Choline	**3.21** (CH_3, s); **3.52** ($^{1}CH_2$, m); **4.07** ($^{2}CH_2$, m)	**56.8** (CH_3); **70.3** ($^{1}CH_2$); **58.5** ($^{2}CH_2$)
10	Ethanolamine	**3.15** ($^{1}CH_2$, t); 3.8 ($^{2}CH_2$, t)	**44.1** ($^{1}CH_2$, t); 60 ($^{2}CH_2$, t);
11	Formate	**8.46** (CH, s)	174 (CH)
12	Fructose	**3.57, 3.72** ($^{1}CH_2$, dd); **3.70, 4.03** ($^{6}CH_2$, dd); **3.80** (^{3}CH, d); 3.8,**4.00** (^{5}CH, d); **3.90**, **4.12** (^{4}CH, t)	**65.3** ($^{1}CH_2$); **66.2** ($^{6}CH_2$); **70.5** (^{3}CH); 70 (^{5}CH); **72.5** (^{4}CH)
13	Glucose	**3.25** ($^{2\beta}$CH, dd), **3.41** (^{4}CH, dd); **3.47** ($^{5\beta}$CH, m); **3.50** ($^{3\beta}$CH, t); **3.54** ($^{2\alpha}$CH, dd); **3.72** ($^{3\alpha}$CH, t); **3.73** ($^{6\beta}CH_2$, dd), **3.84** ($^{5\alpha}$CH, m); **3.90** ($^{6\alpha}CH_2$, dd); **4.65** ($^{1\alpha}$CH, d); **5.24** ($^{1\beta}$CH,d)	**77.0** ($^{2\beta}$CH); **72.5** (^{4}CH); **78.8** ($^{5\beta}$CH); **78.7** ($^{3\beta}$CH); **74.3** ($^{2\alpha}$CH); **75.7** ($^{3\alpha}$CH); **63.7** (^{6}CH); **74.4** ($^{5\alpha}$CH); **98.8** ($^{1\alpha}$CH); **94.9** ($^{1\beta}$CH)
14	Glutamate	**2.07, 2.14** (H^{β},m); **2.36** (H^{γ},m); **3.76** (H^{α},t)	**29.8** (C^{β}); **36.4** (C^{γ}); **57.4** (C^{α})
15	Glutamine	**2.16** (H^{β},m); 2.5 (H^{γ},m); **3.78** (H^{α},t)	**29.1** (C^{β}); 34 (C^{γ}); **57.2** (C^{α})
16	Guanidoacetate	**3.79** (CH_2, s)	**46.3** (CH_2, s)
17	Histamine	3.0 ($^{2}CH_2$, t);3.3 ($^{1}CH_2$, t); **7.10** (^{2}CH, s); **7.86**(^{1}CH,s)	26 ($^{2}CH_2$, t); 41($^{1}CH_2$, t); 120 (^{2}CH, s); 140 (^{1}CH,s)
18	Histidine	3.2, 3.2 (H^{β},dd); 4.0 (H^{α},t); **7.10** (H^{δ},s); **7.84** (H^{ε},s)	31 (C^{β}); 58 (C^{α}); 120 (C^{δ}); 139 (C^{ε})
19	Isoleucine	**0.94** (H^{δ},t), **1.02** (H^{γ},d); 1.2, 1.5 (H^{γ},m); 2.0 (H^{β},m); 3.7 (H^{α},d)	**14.0** (C^{δ}); **17, 6** (C^{γ}); 39 (C^{β}); 62 (C^{α})
20	Lactate	**1.33** (CH_3, d); 4.1 (CH, q)	**22.3** (CH_3); 71 (CH)
21	Leucine	**0.96**, **0.97** (H^{δ},d); 1.7 (H^{γ},m); 1.7 (H^{β},m); 3.7 (H^{α},m)	**23.8**, **24.8** (C^{δ}); 27 (C^{γ}); 43 (C^{β}); 56 (C^{α})
22	Lysine	1.4 (H^{γ},m); **1.73** (H^{δ},m); **1.92** (H^{β},m); **3.03** (H^{ε},t); 3.7 (H^{α},t)	24 (C^{γ}); **29.3** (C^{δ}); **32.7** (C^{β}); **42.0** (C^{ε}); 57 (C^{α})
23	Malate	**2.37, 2.67** (CH_2, dd); **4.30** (CH, dd)	**45.5** (CH_2); **73.3** (CH)
24	Malonate	**3.15** (CH_2, s)	50 (CH_2)
	Methanol (contaminant)	**3.36** (CH_3, s)	51 (CH_3, s)
25	Oxalacetate	**2.41** (CH_2, s)	**28.4** (CH_2)
26	Phenylalanine	**3.12, 3.29** (H^{β},dq); 3.9 (H^{α},q); **7.34** (H^{δ},d); **7.39** (H^{ζ} ,t); **7.43** (H^{ε},t)	42 (C^{β}); 60 (C^{α}); 132 (C^{δ}); 131 (H^{ζ},t); 132 (H^{ε},t);

27	Pyroglutamate	**2.04, 2.51** (H^{β},m); **2.41** (H^{γ},t); **4.18** (H^{α},q)	**28.2** (C^{β});**32.5**(C^{γ}); **61.2** (C^{α})
28	Raffinose[a]	**5.44** ($^{1'}$CH, d); **5.00** ($^{1''}$CH, d)**00**	**95.0** ($^{1'}$CH); **101.00** ($^{1''}$CH)
29	Sarcosine	**2.76** (CH_3, s); 3.6 (CH_2, s)	35 (CH_3); 53 (CH_2, s)
30	Succinate	**2.40** (CH_2, s)	**37.1** (CH_2)
31	Sucrose	**3.49** (^{3}CH, t); 3.6 (^{2}CH, dd); **3.69** ($^{1'}CH_2$, s); **3.77** (^{4}CH, t); **3.85** (^{5}CH, m); **3.83** ($^{6,6'}CH_2$, m); **3.90** ($^{5'}$CH, m); **4.06** ($^{4'}$CH, t); **4.23** ($^{3'}$CH, d); **5.42** (^{1}CH, d)	**72.2** (^{3}CH); 74 (^{2}CH); **64.3** ($^{1'}CH_2$); **75.6** (^{4}CH); **75.3** (^{5}CH); **65.4** ($^{6,6'}CH_2$); **84.2** ($^{5'}$CH); **76.9** ($^{4'}$CH); **79.4** ($^{3'}$CH); **94.9** (^{1}CH)
32	Tartrate	**4.35** (CH, s)	77 (CH)
33	Threonine	**1.33** (H^{γ},d); 4.3 (H^{β},m); 3.6 (H^{α},d)	**22.3** (C^{γ}); 69 (C^{β}); 63 (C^{α})
34	Trimethylamine functional group[b]	**3.22** (CH_3, s); **3.225** (CH_3, s); **3.23** (CH_3, s)	**56.9** (CH_3)
35	Tyrosine	**3.07, 3.18** (H^{β},dq); 3.9 (H^{α},q); **6.91** (H^{ε},d); **7.20** (H^{δ},d)	42 (C^{β}); 60 (C^{α});120 (C^{ε}); 134 (C^{δ})
36	Valine	**1.00, 1.05** (H^{γ},d); 2.3 (H^{β},m); 3.6 (H^{α},d)	**19.6, 20.8** (C^{γ}); 32 (C^{β}); 63 (C^{α})

[a]The full list of chemical shifts for the trisaccharide raffinose is not listed here; however, the assignment was based on the values of the two characteristic anomeric carbons of the two 6-membered rings; bThe chemical shifts listed are for the trimethylamine group of metabolites such as o-acetylcholine, o-phosphocholine, carnitine, sn-glycero-3-phosphocholine, etc.

and Crimson Sweet. NMR data were analyzed using multivariate statistical analysis. PCA score plots showed distinct differences in metabolite profiles between the *CA* PIs and watermelon cultivars. Using 1D ^{1}H and 2D ^{1}H–^{13}C NMR spectroscopy, 36 metabolites that are common to all samples were identified (Table 2).

A consensus of metabolites that were significantly higher in *CA* PI's compared with the cultivars is listed in Table 3. Among the *CA* PIs, PI 189225 (1832) and PI 482324 (1849) showed the most significant metabolic differences in relation to Charleston Gray and Crimson Sweet, respectively. Since every *CA* PI was compared with Charleston Gray and Crimson Sweet, the Mahalanobis (D_M) distance was used to quantify the distance between group centroids, thus indicating the magnitude of the metabolic profile separation. The statistical significance of the cluster separations was assessed by calculating the Hotelling's T^2 two-sample statistic, relating this statistic to an *F*-value, and then applying an *F*-test. If the *F* true value was higher than the *F* critical value then D_M shows a statistically significant separation between the groups (Table 4 and Fig. 1). A clear separation was observed between the *CA* PIs and the watermelon cultivars (*CL*), coinciding with the wide genetic diversity that exists between the two *Citrullus* spp. The wide metabolic variation between the *CA* PIs coincides with the wide genetic diversity among PIs in this *Citrullus* species which exists in diverse geographical regions, including savannas and bushman lands in southern Africa.

A statistical significance was confirmed between all of the pair-wise comparisons. Statistically significant metabolite differences contributed to the score plot separations and were identified by using loading and volcano plots. The significant metabolite fold change analysis for the seven *CA* PIs versus Charleston Gray indicated that choline, malate, glucose, raffinose, and UNK 1 (depending on the pair-wise analysis) content were significantly higher in concentration in the *CA* PIs. Yet, asparagine and metabolites containing the trimethylamine functional group were unique to PI 189225 (1832). The unidentified peaks at 7.68, 7.64, and 6.41 ppm (Supplemental Fig. 1) and glutamine were significantly higher in five out of seven *CA* PIs, followed by fructose which is higher in four out of seven *CA* PIs. Since PI 189225 (1832) showed the highest separation when compared with "Charleston Gray," the fold changes were analyzed (determined using volcano plots). Normalized bucket intensities and the corresponding metabolites, including asparagine, valine, glutamine, compounds containing the trimethylamine functional group, isoleucine, arginine, glutamate, ethanolamine, and choline were in significantly higher concentration in PI 189225 (1832) roots compared with "Charleston Gray." On the other hand, several metabolites (including the simple sugars glucose and malate) are in significantly higher concentrations in Charleston Gray compared with the *CA* PI 482324 (1849).

Twelve metabolites are in significantly higher concentrations in "Charleston Gray" compared with PI 189225 (1832). These metabolites include arginine, asparagine, choline, ethanolamine, glutamate, glutamine, isoleucine, compounds containing the trimethylamine functional group, valine, and the unidentified peaks at 6.41, 7.64, and 7.68 ppm (Table 5 and Supplemental Fig. 1).

Table 3. Consensus of metabolites that have significantly higher concentrations in the *Citrullus amarus* PIs compared with the *Citrullus lanatus* cultivars.

CA PI versus cultivars
4-Aminobutyrate
Alanine
Arginine
Asparagine
Ethanolamine
Formate
Fructose
Glutamate
Glutamine
Isoleucine
Succinate/oxalacetate
Unk 6.14
Unk 7.64
Unk 7.68
Valine

Among the *CA* accessions, PI 482324 (1849) has the highest metabolite separation from "Crimson Sweet." The following 17 metabolites are in higher concentrations in PI 482324 (1849): choline, ethanolamine, glutamine, glutamate, valine, isoleucine, malate, fructose, alanine, 4-aminobutyrate, succinate, oxaloacetate, glucose, and unidentified peaks at 7.68, 7.64, 6.41, and 1.22 ppm. The pairwise data comparison indicates that all significantly different metabolites are in higher concentration in *CA* PIs compared with "Crimson Sweet." Overall, 26 metabolites and four unidentified NMR peaks in the aromatic and aliphatic regions were present in significantly higher concentration in *CA* PIs than in watermelon cultivars (Table 5).

Discussion

When considering the many years of domestication and narrow genetic base shared by watermelon cultivars, possibly due to the "Founder effect," major differences between the wild type watermelon and watermelon cultivars were expected. Although "Charleston Gray" and "Crimson Sweet" share a narrow genetic base, they are still two different genotypes; consequently, they are expected to present differences in metabolite content, but not major differences as shown between these two cultivars and the wild type watermelon accessions.

Table 4. Statistical analysis and significance levels of metabolic change in *Citrullus amarus* PIs as compared with *Citrullus lanatus* Crimson Sweet (CSW) and Charleston Gray (CG).

Statistical Parameter	1254 vs. CSW	1446 vs. CSW	1482 vs. CSW	1813 vs. CSW	1832 vs. CSW	1849 vs. CSW	2001 vs. CSW
D_M	4.43	4.17	4.57	3.76	7.35	14.94	2.43
F-true	26.48	23.49	28.24	19.09	72.91	301.50	7.95
F-critical	4.96	4.96	4.96	4.96	4.96	4.96	4.96
Significance status	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Statistical Parameter	1254 vs. CG	1446 vs. CG	1482 vs. CG	1813 vs. CG	1832 vs. CG	1849 vs. CG	2001 vs. CG
D_M	6.19	3.41	4.12	5.51	7.14	4.90	5.27
F-true	51.74	15.73	22.93	41.02	68.76	32.37	37.48
F-critical	4.96	4.96	4.96	4.96	4.96	4.96	4.96
Significance status	Yes	Yes	Yes	Yes	Yes	Yes	Yes

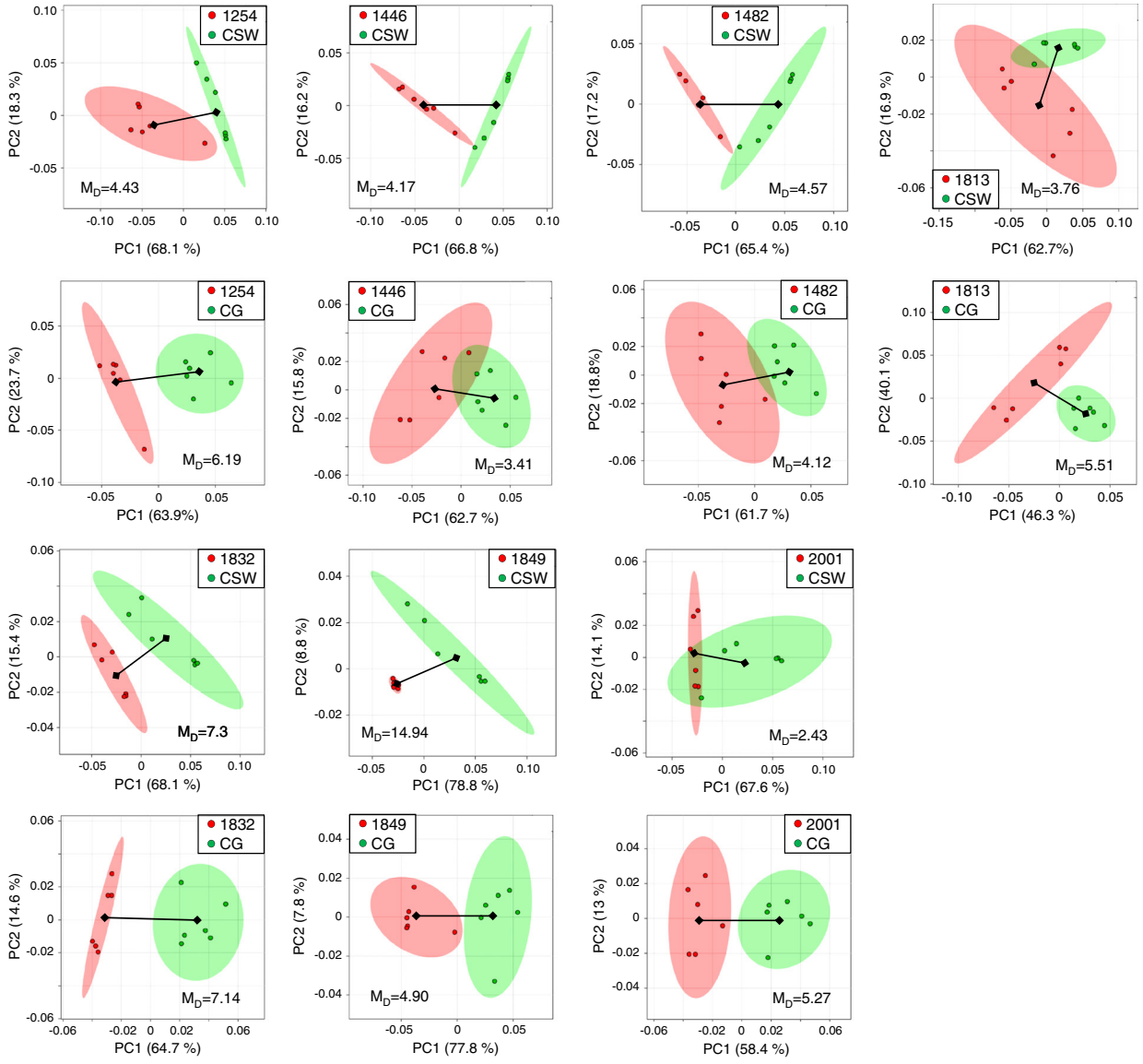

Figure 1: Pair-wise comparison between *Citrullus amarus* PIs and *Citrullus lanatus* Charleston Gray (CG), respectively Crimson Sweet (CSW). PC1 vs. PC2 score plots are shown for each pairwise comparison with a total explained variance of >70% for each. The ovals represent 95% confidence intervals, and the Mahalanobis distances (M_D) are listed for each.

The 1D ^{1}H NMR metabolomics profile analysis along with the biochemical pathway analysis was performed to elucidate the metabolite differences between *CA* and the *CL* watermelon cultivars. In addition to the 1D ^{1}H NMR, 2D ^{1}H–^{13}C HSQC spectra was also performed to verify the ^{1}H assignments (Mahmud et al., 2014). The metabolic differences in this study between *CA* and *CL* could be the result of years of evolution between these two *Citrullus* spp. The *CA* PIs were collected in the wild in southern Africa. The *CL* is endemic to arid or semi-arid regions in northern Africa and gave rise to sweet dessert watermelon which has undergone domestication and selection for desirable fruit qualities while likely losing alleles associated with resistance to biotic and abiotic stress (Levi et al., 2001).

Plants produce diverse secondary compounds that are not directly associated with growth or reproduction, but play an important role in adaptation of plant species to their environment (War et al., 2012). Secondary metabolites play an important role in plant response to biotic and abiotic stress (Facchini, 1999) and are derived from a few primary metabolic compounds that take part in the metabolic pathways.

Table 5. Significantly different metabolites in each of the *Citrullus amarus* PIs when compared with *Citrullus lanatus* Charleston Gray (CG), respectively Crimson Sweet (CSW).

	1832	1254	1849	1813	1446	1482	2001
Metabolites with higher concentrations in resistant lines[a]	**Crimson Sweet (CSW), Fold Change or Charleston Grey (CG), Fold Change**						
2-oxoglutarate + 4 -aminobutyrate	–	–	–	–	–	CSW 6.27	–
4-aminobutyrate	–	–	CSW 5.42	–	–	–	CSW 6.23 CG 1.66
Alanine	CSW 3.14	–	CSW 4.53 CG 4.56	–	CSW 2.11	CSW 4.02 CG 1.53	CSW 4.43 CG 1.78
Arginine	CSW 3.4 CG 2.50	–	–	–	–	–	–
Asparagine	CSW 47.72 CG 21.47	–	–	–	–	–	–
Betaine	CSW 5.87	–	–	–	–	–	–
Choline	CSW 2.86 CG 1.76	–	CSW 2.40 CG 1.50	–	CSW 1.63	–	CSW 2.32 CG 1.41
Ethanolamine	CSW 5.77 CG 1.86	–	CSW 4.11	–	–	–	CSW 3.77
Formate	–	–	–	–	CSW 2.51 CG 3.07	–	–
Fructose	–	CSW 2.83 CG 1.97	CSW 2.38 CG 1.85	–	CSW 2.36	CSW 3.83 CG 2.33	CSW 2.79 CG 1.71
Glucose	CSW 4.28	CSW 2.19	CSW 2.10	–	CSW 1.87	CSW 2.82 CG 1.37	CSW 3.81 CG 1.26
Glutamate	CG 2.29	–	CSW 15.60	–	–	–	CSW 26.02
Glutamine	CSW 5.17 CG 3.09	–	CSW 6.77 CG 5.40	–	CSW 3.81 CG 2.32	CSW 3.81 CG 2.22	CSW 3.14 CG 2.18
Isoleucine	CG 2.59	–	CSW 7.18	–	–	–	CSW 8.73 CG 2.55
Lysine	–	–	–	–	–	–	CG 14.09
Malate	–	CSW 20.6 CG 1.75	CSW 2.47	CSW 3.58	CSW 21.94 CG 1.97	CSW 9.79 CG 1.61	–
Malonate + ethanolamine	–	–	–	–	–	CSW 2.29	–
Multiple[b]	CSW 2.27	–	CSW 2.16	–	–	–	CSW 2.37
Raffinose	–	–	–	–	–	–	CSW 2.60 CG 2.22
Succinate	–	CSW 1.94	–	–	CSW 3.23 CG 2.04	–	–

Succinate/ Oxalacetate	–	–	CSW 1.92	–	–	CSW 1.89	–
Sucrose	–	–	–	–	–	CG 2.96	CSW 4.04 CG 3.67
Sucrose/Choline	–	–	–	–	–	CSW 2.95	–
Tartaric acid	–	–	–	–	–	CSW 2.20	–
Trimethylamine functional group	CG 2.72	–	CG 1.30			CSW 1.86	CG 1.71
UNK at 1.22	–	–	CSW 1.88	–	–	–	–
UNK at 6.41	CG 5.07	–	CSW 7.34 CG 7.49	CSW 5.94 CG 6.12	–	CSW 5.05 CG 4.92	CSW 4.94 CG 5.05
UNK at 7.64	CSW 4.28 CG 5.40	–	CSW 7.08 CG 8.24	CG 4.82	–	CSW 4.20 CG 5.00	CSW 4.32 CG 5.52
UNK at 7.68	CG 3.61	–	CSW 2.08 CG 3.49	CSW 4.89 CG 4.12	CSW 1.91	CG 2.21	CG 3.02
Valine	CSW 9.94 CG 3.58	–	CSW 9.47	–	–	CSW 5.33 CG 3.07	CSW 10.22 CG 2.59

[a]Metabolites joined by a "+" indicate the presence of both at the specific chemical shift used to calculate the fold change, while those joined by a "/" indicate ambiguity; bMultiple = Raffinose + Choline + Sucrose + Fructose + Glucose.

Plants from wild sources often contain higher concentrations of secondary metabolites than their counterpart cultivars. For example, wild raspberries (*Rubus idaeus* L.) (Çekiç and Özgen, 2010), wild fennel (*Foeniculum vulgare* Mill.) seeds, and wild laurel (*Laurus nobilis* L.) leaves (Conforti et al., 2006) have higher polyphenol concentrations than popular cultivated crops. Lower concentrations of secondary metabolites observed in domesticated plants is likely the result of selection for desirable horticultural qualities but not for secondary metabolites that play an important role in plant defense against biotic or abiotic stress (Hadacek and Günther, 2002). Chitwood (1993, 2002) elucidated several groups of metabolites associated with resistance against nematodes, including polythienyls, alkaloids, phenolics, polyacetylenes, fatty acids, and terpenoids. It is likely that the resistance of *CA* PIs to RKN (Thies and Levi, 2003, 2007) is the result of a naturally occurring secondary metabolite(s). These metabolites may be in lower concentrations in watermelon cultivars.

The compounds identified in this study were grouped into three categories: amino acids, carbohydrates, and organic compounds based on the data gathered using 1D ^{1}H NMR.

Amino acids

Amino acids have been known to influence the development of nematodes (Sharma, 1998). Some amino acids (such as glutamine) enhance the development of nematodes like *Heterodera schachtii* while others (including methionine, phenylalanine, lysine, and tryptophan) inhibit the development of nematodes (Sharma, 1998; Betka et al., 1991). In this study, different amino acid metabolites such asparagine, arginine, valine, glutamine, isoleucine, alanine, and lysine were identified in higher concentrations in *CA* PIs when compared with both "Charleston Gray" and "Crimson Sweet." The amino acids asparagine and arginine were identified in higher concentrations only in *CA* PI 189225 (1832).

Asparagine concentrations were 21 times higher in PI 189225 (1832) when compared with Charleston Gray and 47 times higher when compared with Crimson Sweet. Statistically significant metabolites were analyzed using MetaboAnalyst3.0 and KEGG Pathway database to identify the significant metabolites' pathways. A KEGG Pathway database search showed that asparagine plays an important role in alanine, aspartate, and glutamate metabolism, all of which are involved in the amino acid metabolism. Aminoacyl-tRNA biosynthesis is another important metabolic pathway and in which asparagine plays an important role in the translation process (Fig. 2).

A closer look at the biochemical pathways associated with asparagine shows that it acts as a precursor for aspartate which is further used in the biosynthesis of arginine (Berg et al., 2002). Arginine is also found in much higher concentration in the same

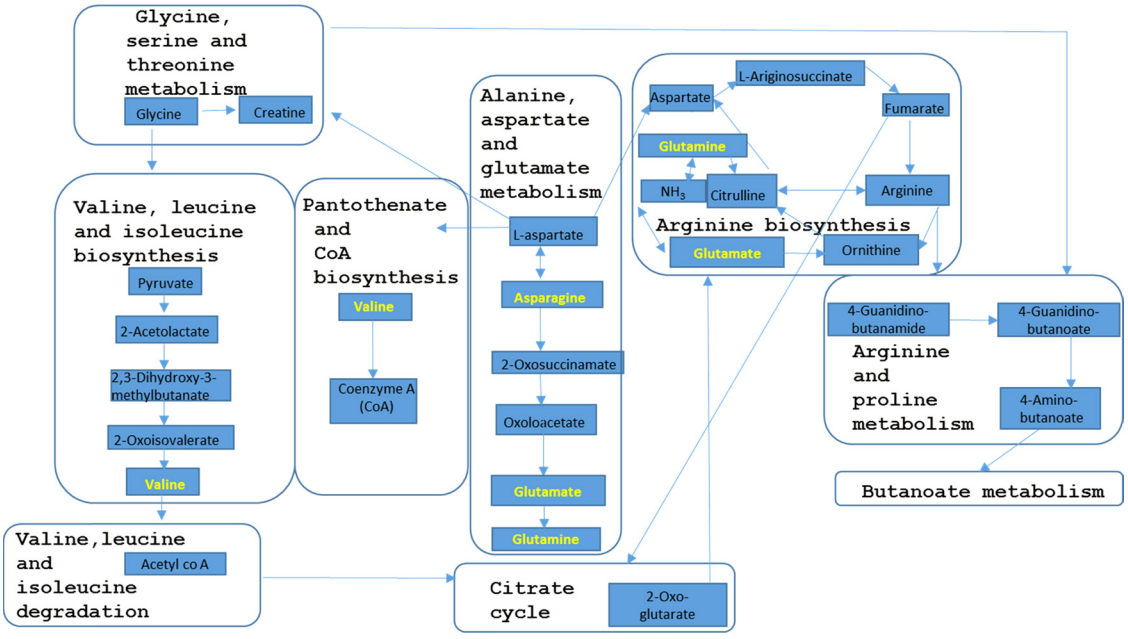

Figure 2: Pathway analysis using significantly different metabolites involved 9 metabolic pathways (shown inside rounded boxes). Yellow highlighted metabolites correspond to those listed in Table 3.

PI 189225 (1832) and it was reported to have a nematicidal effect in a study performed by Sayed and Thomason (1988). That study showed the effectiveness of ascorbic acid, thiamine, arginine, and glutamic acid on egg hatch, development, and reproduction of *Meloidogyne incognita.* Foliar sprays were applied in different concentrations, with their results showing that ascorbic acid, arginine, and glutamic acid suppressed the number of root galls, females, and egg masses. The same study also showed that the highest effect of foliar sprays using ascorbic acid and arginine was observed when the treatment is applied before inoculation with the nematodes. Guo et al. (2013) identified two enzymes (arginosuccinase and two arginosuccinate synthase) that are involved in conversion of citrulline (nonessential amino acid produced from glutamine) into arginine.

Glutamine is another amino acid present in significantly higher concentrations in five PIs in the pair-wise analysis with Charleston Gray and Crimson Sweet. Although it is well known that glutamine can accumulate due to stressful conditions (Hildebrandt et al., 2015), few reports have previously mentioned increased levels of glutamine in different plant tissues as a result of nematodes infection. These studies could not find any nematicidal activity of glutamine. More specifically, sugar beets infected with nematodes showed a significant increase of glutamine, aspartic acid and glutamic acid (Roy, 1981). Similarly, another study performed by Doney et al. (1970) on sugar beets reported an increased concentration in total amino acids (especially in the concentrations of glutamic acid, glutamine, and aspartic acid) in the fibrous roots of infected plants with nematodes.

Another amino acid, valine, was found in higher concentration in three *CA* PIs when compared with Charleston Gray, and in four of the PIs when compared with Crimson Sweet. KEGG metabolite pathway analysis revealed that valine was involved in the following pathways: pantothenate and CoA biosynthesis; valine, leucine, and isoleucine biosynthesis; valine, leucine and isoleucine degradation and; glucosinolate biosynthesis and aminoacyl-tRNA biosynthesis. In the literature, valine was reported to have a significant effect on reducing the number of galls, egg masses, and juvenile nematodes in DL-valine treated tomato plants (Hoque et al., 2014).

Even though there are no reports on the nematicidal activity of different amino acids in watermelon related literature, scientists have already studied the effect of different amino acids on the root-knot nematodes of other plants (tomato) (Hoque et al., 2014). Their results showed that some amino acids, especially DL-phenylalanine, L-proline, and L-histidine, promote plant growth and reduce galling incidence. Leonetti et al. (2010) showed that nitric oxide (NO) and hydrogen peroxide play an important role in tomato plants' defenses against the root-knot nematodes. Nitric oxide is produced by three isoforms of NO synthase and all three isoforms use L-arginine and molecular oxygen as substrates (Forstermann and Sessa, 2012).

Carbohydrates

Once plants are infected with *M. incognita*, more than 50% reduction of free sugars was reported when compared with healthy plants (Roy, 1981). Sugars, especially sucrose, represent the major source of carbohydrate input into nematode-induced feeding sites in *Arabidopsis thaliana* roots and metabolite analysis revealed increased levels of sucrose in syncytia and giant cells (Cabello et al., 2014). Carbohydrates were reported not to have a nematicidal effect; however, fructose, which does not have any nematicidal effect alone, makes DMDP (2R, 5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidine) in combination with rotenone, a highly effective nematode control agent. DMDP can be applied in foliar application and translocated to the plant roots (Paranjape et al., 2014). In this study, fructose was found in higher concentrations in four *amarus* PIs when they were compared with Charleston Gray and in five PIs when they were compared with Crimson Sweet by using metabolite fold change analysis. Using the same metabolite fold change analysis, glucose was identified in higher concentrations in two *CA* PIs (PI 482259 and PI 482303) when they were compared with Charleston Gray and in six PIs when compared with Crimson Sweet. By contrast, glucose was present in Charleston Gray in higher quantities when compared with the three *CA* PIs (PI 189225, PI 482319, and PI 244018). Similarly, sucrose and raffinose were the two other sugars identified as significantly different metabolites when the *CA* PIs were compared with Charleston Gray and Crimson Sweet (Table 5).

Organic compounds

Several organic compounds had higher concentrations in the *amarus* PIs when compared with Charleston Gray, namely: ethanolamine, succinate, 4-aminobutyrate, and formate (Table 5). More specifically, choline was found in significantly higher concentrations in three *CA* PIs and the others only in one PI. When the data comparison looked into the significantly different metabolites in the *CA* PIs when compared with Crimson Sweet, several other organic compounds were also identified in addition to the ones named previously: Although betaine, tartaric acid, malonate, ethanolamine, 2-oxoglutarate, and 4-aminobutyrate were found higher in the PI 1849 when compared with Crimson Sweet, there were not significant when compared with Charleston Gray (Table 5). Although we found conflicting results when compared the concentrations of choline, tartaric acid, and betaine in the PI's versus Charleston Gray, choline was the most significant changing metabolite in three out of seven *CA* PIs when compared with the cultivars. The nematicidal effect of choline was previously reported in a perennial shrub found in the central Sahara region, named arghel (*Solenostemma argel*), which also contains acylated phenolic glycosides, flavonoids, monoterpene and triterpenoid saponin. Extracts from aerial parts (leaves) of argel were reported to be very effective (80%–94% mortality rates after 72 hr exposure) against second stage juveniles of *M. incognita* (Abd El-Aziz, Ezz El-Din, 2007).

Tartaric acid was identified as a significantly different compound in one *CA* PI in the pair-wise analysis with Crimson Sweet. Previously, the nematicidal effect of tartaric acid was also reported by Jang et al. (2016). An exposure of 50 mmol/L of oxalic acid solution cau sed 96% mortality of *M. incognita* J2s at 1 day after treatment, whereas applications of 10 mmol/L resulted in mortality rates of 59.5%.

Lastly, another organic compound that was identified to be a significantly different metabolite when *CA* PIs were compared with Crimson Sweet was betaine. The use of seaweed extract on *Meloidogyne javanica* resulted in significant reduction in egg recovery. The effects of the seaweed extract were similar when betaine components of the seaweed extracts were used (Wu et al., 1998).

In summary, this study identified several metabolites in significantly higher concentrations in *CA* which were previously described in the literature to have nematicidal activity. Fold change analysis showed significant differences among some other compounds that could not be identified using Chenomx or available online databases. This study elucidated the richness of metabolites in roots of *CA* watermelons collected in the wild in southern Africa versus the limited number of metabolites in two major watermelon cultivars. The metabolite diversity between the *CA* PIs may be the result of the high genetic diversity that exists within the *CA* genome. The difference between the *CA* and the cultivars may have resulted from the loss of multiple genes during the domestication process of the watermelon. Despite their wide genetic diversity, *CA* and watermelon cultivars are readily crossable with each other. However, the higher amounts of metabolites in *CA* that have been previously described in the literature to have nematocidal activity could not be directly associated with any effect on nematodes. The *CA* PIs have proved useful as rootstocks for grafted watermelon in fields infested with RKN (Thies et al. 2012, 2015a, 2015b, 2015c), and are considered a useful germplasm for enhancing resistance to RKN and other soilborne diseases in watermelon (Thies and Levi, 2003, 2007). Thus, further studies are underway to determine if any of the statistically significant metabolites that were

present in *CA* PIs have nematicidal effects that might be related to the *CA* PIs' resistance to RKN.

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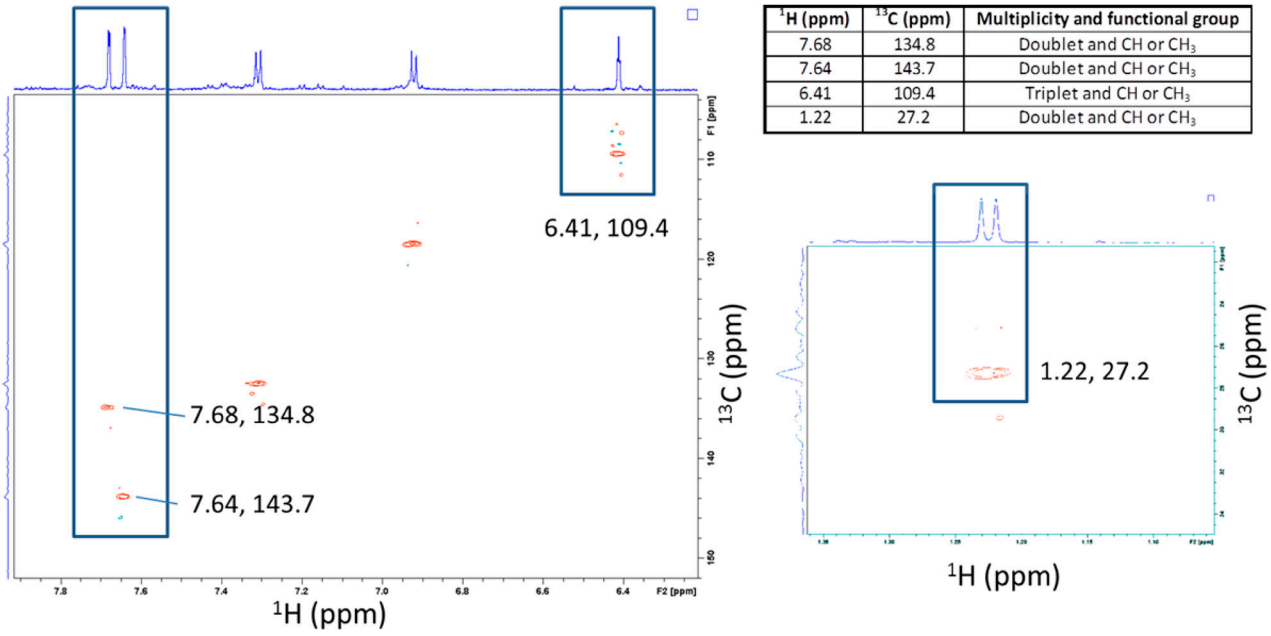
Appendix

Supplemental Fig 1.



^{1}H (ppm)	^{13}C (ppm)	Multiplicity and functional group
7.68	134.8	Doublet and CH or CH_3
7.64	143.7	Doublet and CH or CH_3
6.41	109.4	Triplet and CH or CH_3
1.22	27.2	Doublet and CH or CH_3

Supplemental Figure 1: Unknown peak assignments using one dimensional ^{1}H and two dimensional ^{1}H–^{13}C HSQC NMR. The ^{1}H spectrum is shown in blue and is at the top of the ^{1}H–^{13}C HSQC shown with red contour points. Each contour point of the HSQC represents a hydrogen directly bounded to a carbon. This specific HSQC experiment shows red contour points for CH or CH_3 correlations and green contour points for CH_2 correlations.