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RESEARCH PAPER



Phenolics, flavonoids and antioxidant capacities in *Citrus* species with different degree of tolerance to Huanglongbing

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ABSTRACT

Huanglongbing (HLB) is a highly destructive disease to the citrus industry in Florida caused by the bacterium, *Candidatus Liberibacter asiaticus* (CLAs) and is transmitted by *Diaphorina citri*. It is hypothesized that plants with high phenolic contents show higher tolerance to certain plant pathogens. In this regard, different citrus varieties and plants of genera related to *Citrus* were analyzed for their total phenolic and flavonoids contents, and their antioxidant capacities. In addition, the secondary metabolites in the leaves of seven citrus species were analyzed using high performance liquid chromatography-mass spectrometry (HPLC-MS). Colorimetric assays showed that curry leaf contained the highest total phenolic content and free radical scavenging activity (DPPH). Curry leaf also contained high concentrations of an unusual class of carbazole alkaloids. Tolerant *Citrus* species contained high levels of phenolics and flavonoids and showed high antioxidant capacities. Our results suggest that high phenolic and flavonoid leaf contents correlate with increased citrus tolerance to CLAs bacterium. The results also suggest that the high level of carbazole alkaloids, known for their strong antimicrobial properties in curry leaf, could make it immune to the CLAs bacteria. Understanding the mechanisms underpinning citrus tolerance to HLB will contribute to the development of commercially tolerant citrus cultivars.

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Introduction

In North America, Huanglongbing (HLB) is caused by the bacterium *Candidatus Liberibacter asiaticus* (CLAs) and is transmitted by the Asian citrus psyllid, *Diaphorina citri*. Currently, there is no cure for the HLB disease and its management mainly depends on insecticides to control the insect vector (*D. citri*).¹ Several other management strategies including enhanced nutritional programs, thermotherapy, and removal of infected trees have been tested for the control of HLB; however, these strategies have usually shown poor results in the field.² For these reasons, the development of CLAs-resistant citrus cultivars might be the best means for the control of the HLB disease.

Field observations showed that *Murraya paniculata* (L.) Jack (orange jasmine) and sweet oranges were the most preferred host for *D. citri*, while *Poncirus trifoliata* was a poor host.³ A greenhouse-controlled study showed that grapefruit was a more preferable host than *Citrus jambhiri* Lushington (rough lemon), *Murraya paniculata*, and *Citrus aurantium* L. (sour orange).⁴ Similarly, it was found that *D. citri* preferred *Citrus macrophylla* Wester (Alemow) more than accessions of *Poncirus trifoliata* x *Citroncirus* spp.⁵ Fewer eggs were laid on *P. trifoliata* accessions than *C. macrophylla*.⁵ The resistance of *P. trifoliata* to *D. citri* was attributed to the effect of antixenosis (deterrents) and antibiosis (antagonistic association).⁵

In a similar manner, field observations have shown that some citrus cultivars are more tolerant to the HLB pathogen than others.³ Using graft inoculation, Folimonova et al. (2009) studied the responses of several citrus species to CLAs, and in agreement with the previous reports, certain tested species showed different degrees of tolerance to CLAs. For example, *Poncirus trifoliata*, Eureka lemon (*C. limonia* Osbeck) and *Citrus latipes* were tolerant to the CLAs bacterium, whereas sweet oranges and grapefruits were very sensitive.⁶ The sensitive species showed severe leaf chlorosis, decreased growth and death, whereas tolerant cultivars showed little or no symptoms. In a similar study, Malaysian citrus species also showed different degrees of tolerance to CLAs pathogen.⁷

Unfortunately, it is not clear why are some citrus species are more tolerant to CLAs than others. In order to examine possible mechanisms underpinning the tolerance of some cultivars, we investigated the metabolite profiles of several cultivars of citrus.^{8,9} Our results showed that tolerant cultivars contained higher levels of metabolites that possess strong antimicrobial activities and compounds implicated in plant defense.^{8,9} In another study, we analyzed the metabolite profiles of 'Sugar Belle' mandarin and four of its parents in order to understand why 'Sugar Belle' was more tolerant to HLB.¹⁰ Our metabolomic analysis showed that "Sugar Belle" mandarin was high in thymol, which has a strong anti-fungal and antimicrobial activity.¹⁰ 'Sugar Belle' was also high in several other polar compounds including benzoic acid, ferulic acid,

caffeic acid, synephrine, and inositol, which may protect it from biotic and abiotic stress.¹⁰ We also showed that the metabolomic profile of Australian finger lime, a highly tolerant *Citrus* relative, was different from that of ‘Sugar Belle’, indicating that they could have different tolerance mechanisms.¹¹

Plants produce a wide variety of secondary metabolites that can act as antimicrobial substances such as alkaloids, flavonoids, and phenols. Phenolics are a group of secondary metabolites, which are produced via the shikimic acid pathway through the phenylpropanoid pathways.¹² It is believed that accumulation of phenolic compounds at the infection site could result in isolation of the attacking pathogens and prevent their spread to other plant’s tissues.¹³ In addition, crosslinking of phenylpropanoid esters results in the formation of lignin-like polymers, which leads to the stiffening of the plant cell wall.¹³ Previous reports also showed that apple resistance to *Venturia inaequalis* depends on the regulatory genes of phenol synthesis.¹⁴ The total phenolics and shikimic acid contents in *Venturia inaequalis*-tolerant cultivars are higher than susceptible apple cultivars.¹⁵

Flavonoids are widely distributed in plants and they are synthesized in the cytosol through the phenylpropanoid pathway by a set of enzymes.^{16,17} Previous reports showed that flavonoids were implicated in plant resistance to biotic (herbivory and nematodes) and abiotic stresses including drought, cold, UV-radiation, and toxic metals such as aluminum.¹⁶ Flavonoids are also implicated in plant resistance to fungal and microbial attacks. Flavonoids could exhibit their resistance to pathogens by inhibition and crosslinking of the microbial enzymes, chelation of metals necessary for enzyme activity, and formation of physical barrier.¹⁶

The total phenolic content is normally measured using the Folin-Ciocalteu method, which is based on the reaction between Folin-Ciocalteu reagent with reductants such as phenols, resulting in blue color formation.¹⁸ Yet a major limitation to this assay is its lack of specificity and the results

of this assay cannot be unequivocally attributed to a single class of reductants (i.e., phenolics) in biological tissues. Flavonoids are generally estimated using aluminum chloride, which forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonol.¹⁹ Yet, in complex plant extracts, even this method can show responses to compounds unrelated to flavones and flavonoids. The antioxidant activity is normally estimated using the DPPH assay, which is based on the ability of antioxidants to transfer an electron or a hydrogen to the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).^{20,21}

In this study, we investigated the total phenolic and flavonoid contents as well as the antioxidant and reducing activity of 21 citrus species and two *Citrus* relatives. We hypothesize that CLas-tolerant cultivars contain higher amounts of phenolic and flavonoid compounds and that they will have higher antioxidant and/or reducing activity compared to susceptible cultivars. Furthermore, we investigated the secondary metabolites of a representative subclass of these species covering a wide range of CLas tolerance. This was done with the intention to better understand the mechanisms behind citrus tolerance to HLB and to help citrus breeders to develop commercially tolerant citrus cultivars.

Materials and methods

Plant materials

Healthy seedlings from 24 citrus cultivars and *Citrus* relatives were used in this study (Table 1). Seeds were purchased from Lyn Citrus Seed, Inc. (Arvin, CA, USA) and individually potted in square plastic pots (30 × 10 × 10 cm) containing Sungro professional growing mix (Sungro Horticulture, Agawam, MA). Germinated seedlings were kept in a greenhouse (28 ± 1°C, 60 ± 5% relative humidity, L16:D8 h photoperiod) at the Citrus Research and Education Center (CREC), University of Florida,

Table 1. Citrus species and citrus relatives used in this study and their degree of tolerance to CLas.

	Species used in this study	Degree of tolerance to CLas
1	Valencia sweet orange (<i>C. sinensis</i> (L.) Osbeck)	1 ^a
2	Madam Vinous sweet orange (<i>C. sinensis</i> (L.) Osbeck)	1 ^a
3	Hamlin sweet orange (<i>C. sinensis</i> L. Osbeck)	1 ^a
4	Duncan grapefruit (<i>C. paradisi</i> MacFadyen)	1 ^a
5	Ruby red grapefruit (<i>C. paradisi</i> MacFadyen)	1 ^a
6	Sour orange (<i>C. aurantium</i> L.)	2 ^a
7	<i>C. macrophylla</i> Wester (Alemow)	2 ^a
8	Mexican lime (<i>C. aurantifolia</i> (Christm.))	2 ^a
9	Carrizo citrange (<i>X Citroncirus webberii</i>)	3 ^a
10	<i>Citrus latipes</i>	3 ^a
11	Minneola tangelo	1 ^a
12	Citron (<i>Citrus medica</i>)	2 ^a
13	Cleopatra mandarin	1 ^a
14	Sun Chu Sha Kat mandarin (<i>C. reticulata</i> Blanco RUTACEAE)	2 ^b
15	Orange jasmine (<i>Murraya paniculata</i>)	1 ^b
16	Pineapple sweet orange	1 ^b
17	Sugar Belle tangerine	2 ^b
18	Finger lime (<i>Citrus australasica</i>)	2 ^b
19	Curry leaf (<i>Murraya koenigii</i> (L.) Spreng)	4 ^b
20	Bearss lime (<i>C. latifolia</i>)	2 ^b
21	Frost Marsh grapefruit (<i>Citrus paradisi</i> MacFadyen)	2 ^b
22	Bingo tangerine ‘LB8-9’	2 ^b
23	Dancy Tangerine	3 ^b

^aFolimonova et al.⁶

^bField and greenhouse observations.

Lake Alfred, Florida. Seedlings were watered twice weekly. All seedlings were about one-year old. Five plants (about one-year-old) were sampled from each variety by collecting three mature leaves from three different locations (top, middle, and bottom).

Extraction of phenolics and flavonoids from plant tissues

Leaves collected from each variety were pooled and ground in liquid nitrogen using a mortar and pestle, and 100 mg of the homogenous sample was transferred to a 2-mL centrifuge tube. One ml of methanol was added to each tube and the sample was vortexed at 750 rpm for 15 min at 20°C using Eppendorf Thermomixer followed by sonication for 15 min. The samples were centrifuged at 12,000 rpm for 10 min at 20°C. The supernatant was transferred into a new tube and kept at -20°C until analysis.

Total Phenolic Content (TPC)

Total phenolics were determined using Folin-Ciocalteu reagents according to Singleton & Rossi (1965).²² Plant extracts or gallic acid standard (20 µl) were mixed with 0.9 mL of Folin-Ciocalteu reagent (10% in water), and then 0.6 mL of sodium carbonate (7.5%, w/v) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 760 nm. Aqueous solutions of known gallic acid concentrations in the range of 100–600 ppm were used for calibration. Results were expressed as mg gallic acid equivalents (GAE) g⁻¹fresh weight (FW).

Total Flavonoid Content (TFC)

Determination of total flavonoids was performed according to the colorimetric assay of Aktumsek et al. (2013) using aluminum chloride.²³ Distilled water (200 µL) was added to 50 µL of the extract in a test tube. A 15 µL portion of 5% sodium nitrite solution was added, followed by 15 µL of 10% aluminum chloride solution. Test tubes were incubated at ambient temperature for 5 min, and then 100 µL of 1 M sodium hydroxide and 1.2 mL of distilled water were added to the mixture. The mixture was vortexed and the absorbance of the resulting pink color was measured at 510 nm. Aqueous solutions of known catechin concentrations in the range of 10–200 ppm were used for calibration and the results were expressed as mg catechin equivalents (CEQ) g⁻¹FW sample.

Free radical scavenging activity (DPPH assay)

DPPH assay was performed according to Brand-Williams et al.²⁴ Briefly, a 1000 µL aliquot of a 0.1 mM of DPPH solution in methanol was added to 25 µl of each extract or Trolox standard (prepared in the range of 20–200 ppm, dissolved in methanol). The mixture was vortexed for 5–10 sec and incubated at room temperature in dark for 30 min and then the absorbance was measured at 515 nm. The results were expressed as mg Trolox g⁻¹ FW.

Chromatographic conditions

The HPLC-MS analysis of the extracts were run on eclipse C18 column (Eclipse 150 mm, 5 µm). The mobile phase was a mixture of 0.5% acetic acid solution (A) and acetonitrile (B) and was run in a linear gradient mode. The start was a 95% (A) that descended to 70% (A) in 40 min. Then to 40% (A) in 20 min and finally to 10% (A) in 2 min and stayed there for 6 min and then back to the initial conditions in 2 min. The HPLC system was equilibrated for 5 min with the initial acidic water mobile phase (95% A) before injecting next sample. All the samples were filtered with a 0.45 µm PTFE filter. The column temperature was set at 25°C.

A 20-µL aliquot of the final leaf extract was analyzed by HPLC-MS using a Waters Alliance 2695 HPLC coupled with a 996 photodiode array detector and a Waters ZQ single quad mass spectrometer. A flow splitter (10-to-1) was used to simultaneously monitor UV and mass spectra of the eluting analytes. UV spectra were monitored between 600 and 240 nm. ZQ parameters were as follows: MS parameters were as follows: ionization mode, ES⁺; capillary voltage 3.0 kV; extractor voltage 5 V; source temperature 100°C; desolvation temperature 225°C; desolvation N₂ flow 465 L/h, cone N₂ flow 70 L/h; scan range *m/z* 50–1000; scan rate 1 scan/s; and cone voltages 20 and 40 V. Analysis of the chromatograms was performed using ZQ calculated mass-extracted total ion chromatograms (TIC) obtained in scanning mode, or in the single ion response mode. To normalize the mass spectrometer response during sequential runs, an internal standard, mangiferin, was used. Data handling was done with the MassLynx software version 4.1.

Phenolics in methanol extract were tentatively identified/classified by their UV and mass spectra. The numbers of compounds in particular classes of phenols can be estimated with reasonable accuracy using the high peak resolution afforded by analytical-reversed-phase of the HPLC. Estimates of the levels of these compounds were obtained by using peak area-to-microgram conversion factors of authentic standards identified in each group. The measured conversion factors for ferulic acid, nobiletin, hesperidin, diosmin, vitexin, marmin, and mahanimbine were used for hydroxycinnamates, polymethoxylated flavones, flavanone glycosides, flavone-O-glycosides, flavone-C-glycosides, coumarins, and carbazole alkaloids, respectively.

Saponification of hydroxycinnamates in citrus leaf extracts

Portions (150 µL of each leaf extract) were combined with 50 µL 4 N KOH and 200 µL 2 N KOH. The samples were vortexed for 3 min and stored at room temperature for 1 h. The saponified samples were then mixed with 50 µL glacial acetic acid and concentrated HCL until the resulting solutions were adjusted to pH 4.5–6. The final volumes of the samples were brought to 1.0 mL with deionized water. Analytical HPLC using a Waters XBridge C8 column (150 x 4.6 mm), using the same solvent gradients as described above, was used to measure the amounts of liberated hydroxycinnamic acids.

Statistical analysis

Data were analyzed using JMP 9.0 software (SAS, Cary, NC). Analysis of variance (ANOVA) followed by post hoc pairwise comparisons using Tukey–Kramer honestly significant different test (Tukey HSD) were used to compare the studied parameters (TPC, TFC, DPPH, and hydroxycinnamates) among the selected varieties.

Results

Total phenolic content

The colorimetric Folin-Ciocalteu assay showed the highest total phenolic content (13.68 ± 1.71 mg GA/g) in the leaves of *Murraya koenigii* (curry) followed by Dancy tangerine (12.55 ± 0.93 mg GA g^{-1} FW), Carrizo citrange (11.15 ± 2.00 mg GA g^{-1} FW), and 'LB8-9', Bingo (10.55 ± 1.94 mg GA g^{-1} FW) (Figure 1(a)). The Tukey's test showed that the level of total phenolic content in curry leaf was significantly higher than all other cultivars (Figure 1(a)). The lowest phenolic content occurred in Duncan grapefruit (4.11 ± 0.57 mg GA g^{-1} FW), ruby red grapefruit (4.62 ± 0.26 mg GA g^{-1} FW), and *C. macrophylla* (4.79 ± 0.53 mg GA g^{-1} FW) leaves (Figure 1(a)). The Tukey's test showed that the level of total phenolic content in Duncan grapefruit was significantly lower than all other cultivars. The rest of cultivars contained moderate levels of phenolic content between 5.31 and 9.37 mg GA g^{-1} FW (Figure 1(a)).

Total flavonoid content

The highest level of flavonoids as measured by the aluminum chloride assay occurred in curry leaf (0.81 ± 0.10 mg g^{-1} FW), followed by Bearss lime, Sunchu mandarin, sour orange, Carrizo citrange, orange jasmine, Bingo, *C. latipes*, and Carrizo citrange (0.49 – 0.43 mg g^{-1} FW) (Figure 1(b)). The level of total flavonoids in curry leaf measured by the aluminum chloride assay was significantly higher than the rest of other plants (Figure 1(b)). The lowest level of flavonoids occurred in Ruby red (0.23 ± 0.018 mg g^{-1} FW) and Minneola tangelo (0.30 ± 0.010 mg g^{-1} FW) (Figure 1(b)). The rest of the cultivars contained moderate levels of flavonoids (with the range of 0.32 – 0.42 mg g^{-1} FW) (Figure 1(b)).

Total antioxidant activity

In agreement with the total phenolic and flavonoids contents, curry leaf was the highest free radical scavenging antioxidant activity DPPH (2.00 ± 0.27 mg g^{-1} FW) (Figure 1(c)). Bearss lime was the second highest plant in DPPH (1.52 ± 0.13 mg g^{-1} FW), followed by Dancy tangerine (1.20 ± 0.24 mg/g), orange jasmine (0.92 ± 0.078 mg g^{-1} FW) and finger lime (0.88 ± 0.19 mg g^{-1} FW) (Figure 1(c)). Ruby red grapefruit had the lowest free radical scavenging activity (0.13 ± 0.051 mg g^{-1} FW) (Figure 1(c)). The rest of the cultivars showed moderate free radical scavenging activity (0.21 – 0.8 mg g^{-1} FW) (Figure 1(c)).

HPLC-MS results

Results of the HPLC-MS analyses of the phenolics in seven of the varieties in this study are summarized in Table 2. The main groups of compounds detected in each variety are shown in Figure 2. The number of compounds detected in each class of plant phenols and the calculated estimates of the sum total amounts in each class are given in Table 2 for leaves of curry, Duncan, and Ruby Red grapefruits, Bearss lime, Carrizo citrange, Valencia orange, and Dancy tangerine leaves. The results in Table 2 show that Dancy tangerine and Bearss lime contained the highest summed values for flavone-*O*-glycosides and flavone-*C*-glycosides. Dancy tangerine contained the highest amounts of polymethoxylated flavonoids (PMFs, and was also the highest in hydroxycinnamates (Table 2). Unique to curry leaf were the high concentration of carbazole alkaloids. Calculations based on the known compound mahanimbine show an average carbazole alkaloid concentration of about 14 mg/g.

Accurate measurements of the total concentrations of ferulic, sinapinic, and *p*-coumaric acid-bound hydroxycinnamates were obtained by HPLC analysis of saponified leaf extracts. Dancy tangerine was shown to contain the highest levels of total bound hydroxycinnamic acids (4.30 mg g^{-1} FW) followed by Carrizo citrange (3.6 mg/g FW), Bearss lime (1.90 mg g^{-1} FW) and curry leaf (1.50 mg g^{-1} FW) (Table 3). Curry leaf was the highest in *P*-coumaric acid followed by Bearss lime (Table 3). Dancy tangerine was the highest in ferulic acid followed by Carrizo citrange (Table 3). The two grapefruit varieties, Ruby Red and Duncan contained the lowest levels of bound hydroxycinnamic acids. Curry leaves differed from the other citrus varieties by having a significantly higher *p*-coumaric acid content (1.30 mg g^{-1} FW) than the other leaf sources.

Discussion

Although field observations and greenhouse studies showed that certain citrus genotypes are more tolerant to HLB than other genotypes, the mechanisms behind this tolerance remained largely unknown. In the broad view, citrus tolerance to HLB could result from resistance to the insect vector or to the plant pathogen. The resistance to the vector insect could result from several reasons including antibiosis or antixenosis, whereas as resistance to the CLas bacterium could result from many different factors, primarily which is the presence of bacteriostatic and bactericidal compounds. In our current investigation, we measured the total phenolic and flavonoid contents as well as the DPPH activity of 23 citrus genotypes to test if any of these criteria correlated closely with citrus tolerance to the CLas bacterium.

Total phenolics, total flavonoids, and antioxidant activities can be measured using several methods with different mechanisms. For example, total flavonoids can be measured by colorimetric methods using aluminum chloride and 2,4-dinitrophenylhydrazine. Among the different classes of flavonoids, the aluminum chloride assay is specific for flavones and flavonols, while the 2,4-dinitrophenylhydrazine method is specific for flavanones.¹⁹ In the same manner, several methods with different mechanisms have been developed to determine the antioxidant activities in plants and

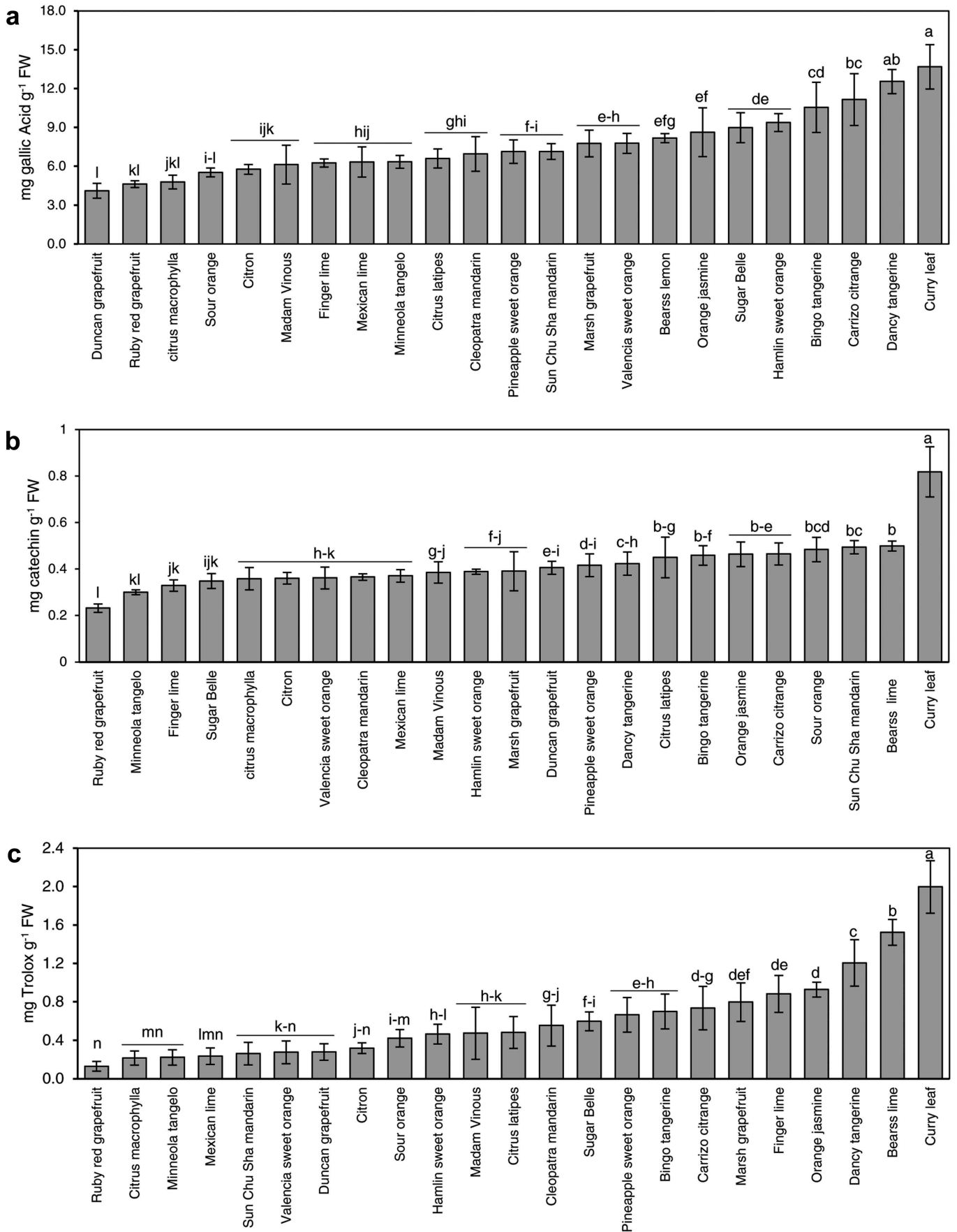


Figure 1. Total phenolics (a), flavonoids (b) contents, and antioxidant activity measured as (DPPH) (c) in selected citrus and citrus relative genotypes. Error bars represent standard deviation ($n = 5$). Varieties with different letters are statistically different using Tukey's HSD test (P -value < 0.05).

Table 2. Concentrations (mgg⁻¹ FW) of main classes of metabolites detected in seven citrus genotypes (*n* = 5).

Species	Chemical group						
	FN-C-glyc	FN-O-glyc	Flavanone	HCA	PMF	Psoralen	Carbazole
Bearss lime	4.73 (10)	11.09 (7)	0.35 (1)	0.19 (2)	0	0.095 (2)	0
Valencia orange	0.91 (7)	1.87 (7)	3.65 (2)	0.10 (1)	1.03 (11)	0	0
Ruby red grapefruit	0.93 (4)	4.17 (6)	2.13 (7)	0.15 (2)	0.04 (3)	0.13(2)	0
Duncan grapefruit	0.11 (2)	1.16 (9)	0.28 (9)	0.031 (2)	0.10 (5)	0.05 (1)	0
Dancy tangerine	4.88 (7)	1.92 (2)	5.46 (2)	4.88 (9)	7.18 (12)	0	0
Carrizo citrange	2.39 (8)	2.27 (4)	3.73 (2)	0.55 (15)	2.66 (6)	0	0
Curry leaf	0	0.59 (4)	0	0.75 (15)	0	0	14.3 (14)

FN-C-glyc: Flavone-C-glycosides, FN-O-glyc: flavone-O-glycosides.

foods.²⁵ Consequently, it is recommended to use more than one method to measure the total antioxidant activities of a biological tissue.²⁵ In this investigation, we measured the total phenolic and flavonoid contents as well as the DPPH activity in the leaves of a wide range of citrus varieties using colorimetric assays, and further analyzed the methanol extract of seven of the selected genotypes by HPLC-MS. To gain an accurate measure of the bound hydroxycinnamic acids in the leaf tissues, the leaf extracts were also saponified and analyzed by HPLC-MS.

The colorimetric assays showed that curry leaf was the highest in total phenolic, total flavonoids, and free radical scavenging antioxidant activity (DPPH). The HPLC-MS analysis showed that curry leaf was high in hydroxycinnamates. However, only four flavonoids were detected and the estimated total flavonoid content in curry leaf was low (0.60 mg g⁻¹ FW). These results indicated that phenolic compounds in curry leaf were interfering with the aluminum chloride assay. In agreement with the HPLC-MS results, saponification of the methanol extracts showed that curry leaf was the highest in *p*-coumaric acid, which has 1.4 Trolox equivalent antioxidant capacity (TEAC).²⁶ Our results were in agreement with previous reports, which showed high phenolic contents as well as high antioxidant capacities in curry leaves.²⁷ In another study, curry leaf was also found to be rich in gallic acid and total phenolic compounds, and showed high DPPH value.²⁸

The HPLC-MS analysis also showed that curry leaf was high in carbazole alkaloid contents. The alkaloid, mahanimbine, and the essential oil of curry leaf showed strong antibacterial activities against several antibiotic-resistant pathogenic bacteria and were effective against several cancer cell lines.²⁹ In a separate study, the methanol extract of curry leaves showed strong antimicrobial activities against several pathogens including *Staphylococcus*, *Streptococcus*, and *E. coli*.³⁰ The antibacterial activities of the curry leaf extract were comparable with gentamycin and amikacin.³⁰ Carbazole compounds from curry leaf also showed high oil stability index and a strong radical scavenging activity against DPPH.³¹ The DPPH radical scavenging activities of most carbazoles were stronger than that of α -tocopherol.³¹ The previous results together indicated that carbazole alkaloids could have a strong antimicrobial activity against CLAs. However, future research is needed to confirm this suggestion.

Our results showed that Dancy tangerine leaves were second highest in total phenolic compounds and the third in antioxidant activity. In agreement with the colorimetric assays, HPLC-MS also showed that Dancy tangerine was rich in hydroxycinnamates, flavanones and flavones, and

polymethoxylated flavones. The saponification results showed that Dancy tangerine was rich in ferulic and sinapic acid. High levels of hydrolyzable hydroxycinnamic acids were earlier found in Dancy tangerine peel.³² Levels of polymethoxylated flavones in Dancy tangerines were several times higher than in most other citrus cultivars.³² Our earlier research showed that several hydroxycinnamates and polymethoxylated flavones were induced in CLAs-infected sweet orange trees.³³ These results suggested that hydroxycinnamates and polymethoxylated flavones could be involved in citrus defense against CLAs. Induction of hydroxycinnamates was also observed in different plants upon bacterial and viral infections.³³⁻³⁵ It is believed that hydroxycinnamates could act as direct antimicrobial agents and signal molecules, and can be deposited into the cell wall to reinforce it against pathogen attack.³⁶ These observations further indicate that Dancy tangerine could be tolerant to the HLB pathogen through its high phenolic content.

Bearss lime was the second in total flavonoids, DPPH, and it contained moderate amounts of total phenolic. In agreement with the colorimetric result, the HPLC-MS results showed that Bearss lime was rich in flavonoids (22 compounds). In addition, the saponification results showed that Bearss lime was the highest in sinapic acid and the fourth in total hydroxycinnamates. These results indicated that it could show some tolerance to the HLB disease.

Previous studies showed that *C. citrange* was relatively tolerant to the CLAs pathogen.⁶ Our current results showed that *C. citrange* was the third highest variety in total phenolic and the fifth in total flavonoids, and the seventh in DPPH. In agreement with these results, the HPLC-MS analysis showed that *C. citrange* was rich in hydroxycinnamates (15 compounds) and it contained moderate amount of flavonoids (20 compounds). Saponification of the methanol extract also showed that *C. citrange* was rich in ferulic acid. Our previous study showed that *C. citrange* was the highest among 13 different varieties in total volatiles and sesquiterpenes and second in total monoterpenes.⁸ With respect to single volatiles, *C. citrange* was the highest cultivar in *t*-caryophyllene, γ -elemene, *d*-limonene, β -elemene, and germacrene D.⁸ In addition, *C. citrange* was the second highest variety in quinic acid.⁹

Flavonoids, which are also considered as a group of phenolic compounds, have a good antioxidant activity.^{26,37} Recently, Zuo et al. (2019) showed that flavones such as luteolin and apigenin and flavonols (quercetin) had potent inhibitory effects on *YbeY* of CLAs bacterium.³⁸ *YbeY* is considered part of the 206 genes that make up the minimal bacterial genome set, which is necessary for the maturation of RNAs. In addition, the *YbeY* portion of the bacterium's genetic code was found to play an important role in

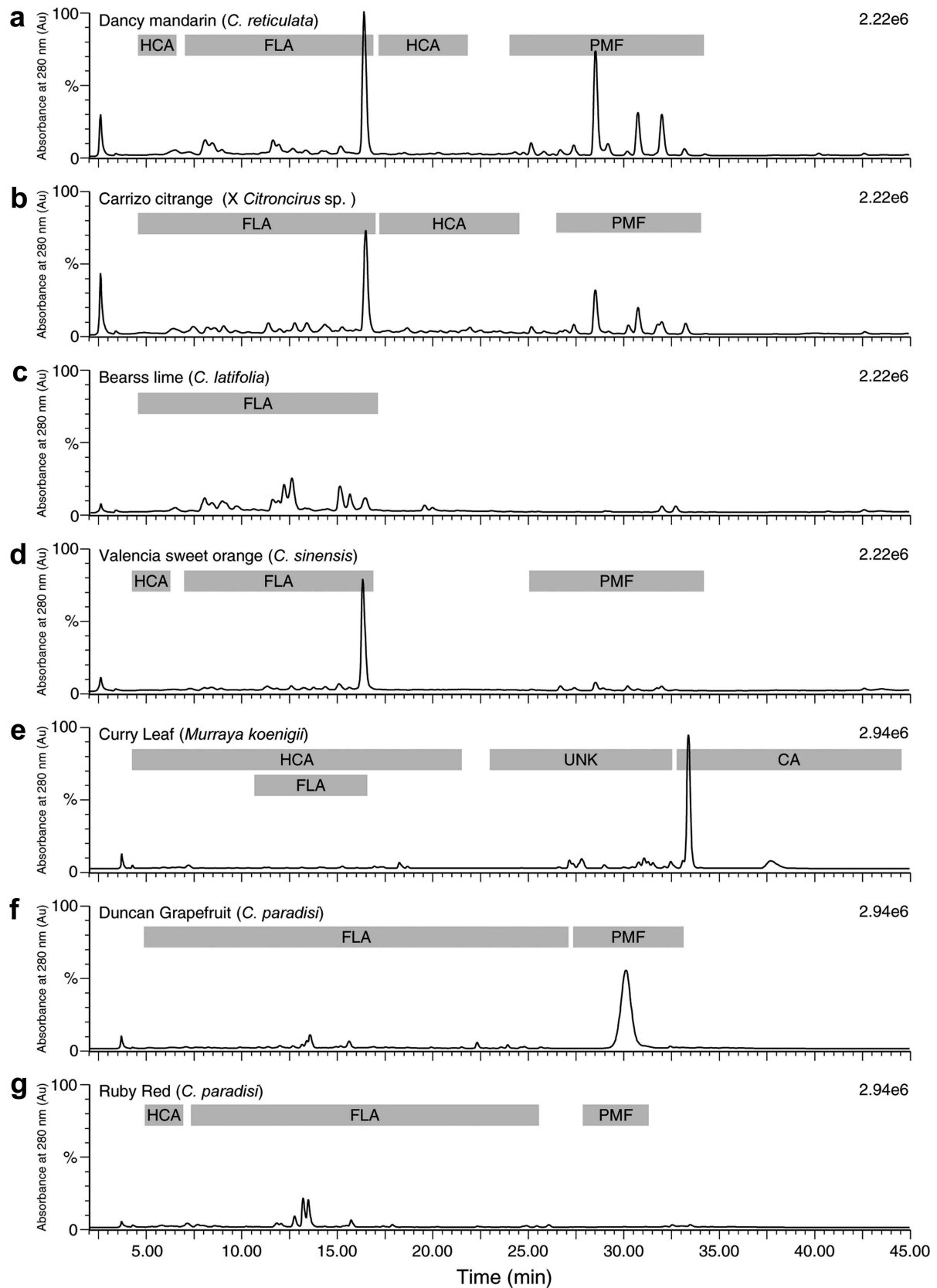


Figure 2. Representative HPLC chromatograms of selected citrus and citrus relative genotypes (a: Dancy mandarin, b: Carrizo citrange, c: Bearss lime, d: Valencia sweet orange, e: Curry leaf, f: Duncan grapefruit, and g: Ruby red) showing main peaks at 280 nm.

Table 3. Concentrations (mg g⁻¹ FW) of hydroxycinnamic acids existing as hydrolysable hydroxycinnamates in seven citrus leaf extracts. Hydroxycinnamic acids measured after leaf extract saponification (*n* = 5).

Species	<i>P</i> -Coumaric	Sinapic	Ferulic
Bearss lime	0.62 ± 0.08 ^b	0.84 ± 0.05 ^a	0.49 ± 0.04 ^b
Valencia orange	0.11 ± 0.01 ^d	0.45 ± 0.04 ^c	0.66 ± 0.38 ^b
Ruby Red grapefruit	0.12 ± 0.01 ^d	0.06 ± 0.01 ^e	0.61 ± 0.05 ^b
Duncan grapefruit	0.11 ± 0.01 ^d	0.03 ± 0.02 ^e	0.60 ± 0.41 ^b
Dancy tangerine	0.28 ± 0.03 ^c	0.57 ± 0.02 ^b	3.44 ± 0.16 ^a
Carrizo citrange	0.26 ± 0.30 ^{cd}	0.32 ± 0.01 ^d	2.98 ± 2.66 ^{ab}
Curry leaf	1.29 ± 0.30 ^a	0.03 ± 0.01 ^e	0.18 ± 0.04 ^c

Varieties with different superscript letters are statistically different using Tukey's HSD test (*P*-value < 0.05).

bacterial symbiosis and pathogenicity.³⁸ Tested flavones and flavonols showed a significant reduction in CLAs titer in HLB-infected citrus trees.³⁸ On the other hand, the flavanones (hesperetin and naringenin) were found to have little to no inhibitory effect.³⁸ The previous results suggested that HLB resistant plants could be generated via genetic or metabolic modification of the biosynthetic pathways of flavones and flavonols.³⁸ Our previous study showed that several flavonoids compounds such as 6,8-di-C-glucosylapigenin, 2''-O-xylosylvitexin, hesperidin, sinensetin, and polymethoxylated flavones were induced in CLAs-infected sweet orange trees.³³ Taken together, these previous results suggest that flavonoids could play a role in citrus defense against CLAs.

Conclusion

Our results showed that tolerant varieties contained higher levels of phenolic compounds and flavonoids than sensitive varieties. Curry leaf, which is immune to CLAs, contained high level of hydroxycinnamates and carbazole alkaloids. Our results suggest that high levels of flavonoids and phenolic compounds might enhance citrus tolerance to HLB. Further studies into the roles of these compounds in imparting tolerance in citrus to the CLAs bacterium may provide useful information for the control of citrus HLB disease.

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