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# Changes in Polyphenol Levels in Satsuma (*Citrus unshiu*) Leaves in Response to Asian Citrus Psyllid Infestation and Water Stress

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**Abstract:** Huanglongbing (HLB), also referred to as citrus greening disease has caused significant losses to the citrus industry in the United States and elsewhere. In our previous studies, we observed the fluctuation of some primary and secondary metabolites in response to biotic (psyllids feeding that transfers bacteria; i.e., *Candidatus* Liberibacter spp. that cause greening disease) and abiotic (water deficit) stress factors in citrus. In the current report, we evaluated the changes in polyphenolic compounds in Satsuma leaves in response to Asian citrus psyllid feeding stress and the water stress. In general, polyphenolic levels increased in Satsuma leaves in response to insect and water stress. Specifically, polyphenols such as chlorogenic acid, rutin, diosmin, luteolin7-*O*-glucoside, and narangin levels increased significantly in response to both biotic and abiotic stress. On the other hand, while caffeic acid levels significantly increased in water stressed plants, their levels drastically declined to the level of being undetectable in leaves stressed by psyllid feeding. Differences between the two types of stresses were also observed in the levels of apigenin 7-O-glucoside where it decreased significantly in water stressed leaves but not in leaves stressed by psyllid feeding; i.e. changes in the levels of apigenin 7-O-glucoside and caffeic acid were opposite in response to water or feeding stresses. Hesperidin levels were not affected by the water stress or by psyllid feeding. The findings may help to better understanding plant psyllid interactions and may be helpful in developing effective management practices to control the spread of citrus greening disease.

**Keywords:** Citrus greening, *Candidatus* Liberibacter, Huanglongbing, Polyphenols, Psyllid.

## INTRODUCTION

Citrus is a multibillion dollar industry in US, including farm production and industrial processing of citrus products, which is now seriously threatened by citrus greening disease [1, 2]. Indeed, citrus greening disease or Huanglongbing (HLB), is considered as one of the most devastating diseases affecting citrus producing countries worldwide [1, 3]. The disease is caused by a phloem inhabiting Gram negative α-Proteobacterium; i.e., '*Candidatus* Liberibacter' species, '*Ca*. Liberibacter asiaticus' (Las), '*Ca*. L. africanus' (Laf) and '*Ca*. L. americanus' (Lam) are associated with the disease [4]. The Asian citrus psyllid (ACP) carries the disease causing bacteria that it transfers to plants when it feeds on phloem of young leaves [2]. There is no known cure for the disease; and therefore, it is primarily managed by controlling the population of the ACP insect vector [5, 6]. Thus, for developing effective management strategies of ACP control it is important to understand biochemistry, especially of secondary metabolites, of ACP- host plant interactions and the effect of abiotic stresses (such as reduced water supply) on the host plant defenses [7].

Plants have evolved mechanisms to cope with both biotic and abiotic stresses, among these are the changes of various primary and secondary metabolites [8-11]. Studies have reported a correlation between water stress and insect attraction, showing water stressed plants may not be as attractive to herbivory as low water stressed plants [12, 13]. Similarly plants have developed mechanisms to respond to herbivory in order to suppress or deter feeding and/or attract natural predators. Plants have been shown to respond to insect stress by producing reactive oxygen species and the induction of defense genes, which in turn leads to the production of defensive metabolites [14]. Phenolic and polyphenolics molecules in plants have been reported to fluctuate in response to insect damage, in-turn resulting in a possible insect management option [15].

Citrus plants endogenously produce many classes of secondary metabolites such as flavonoids, limonoids and simple phenolic compounds [16, 17]. Citrus flavonoids in particular have been reported to possess properties that protect plants

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from various insect insults [18]. For example, naringin and chlorogenic acid have been reported to strongly deter caterpillar feeding in a two choice test leaf assays [19]. Additionally, negative correlations have been reported to occur with increased levels of chlorogenic acid, caffeic acid, and apigenin derivatives and insect feeding in various crops [20, 21]. The levels of metabolites in citrus plants have been reported to fluctuate in response to various biotic and abiotic factors [7, 22]. In our previous publication, we have also reported that a decrease in levels of certain flavonoids in sour orange rootstock plants resulted in an increase of ACP infestation [7]. The maintenance of plants under cold stress conditions resulted in decreased levels of several polyphenolic compounds which consequently led to an increase in ACP infestation [7].

It is, therefore, plausible and important to further understand the influence of various stresses on citrus plants in order to develop possible management practices to deter feeding of ACP on host plants. In this study we evaluated the effect of water stress and ACP feeding changes in secondary metabolite levels found in Satsuma leaves for the first time.

## MATERIALS AND METHODS

### Insects

The Asian citrus psyllids, *Diaphorina citri* Kuwayama, were initially collected from the citrus orchard of Texas AgriLife Research and Texas A&M University System at Weslaco, Texas. The ACP were then continually reared on new flushes of small citrus trees caged in our laboratory facility maintained at 28 ± 2°C and supplemented with light and humidity as described in our previous study [7].

### Plants

Young Satsuma saplings (*Citrus unshiu*; cultivar Owari) purchased from a local nursery were grown at the USDA-ARS facility in 16-liter pots filled with a mixture of peat: perlite: vermiculite (1:1:1 by wt.) and supplied with Peter's nutrient solution at water holding capacity every other week. When these saplings had grown to two year old trees at the USDA-ARS facility (in open field under a 30% shade) in Weslaco, Texas were used for this study; six replicate trees were used for each treatment. One month prior to start of experiment the plants were transported into a testing laboratory (maintained at 28 ± 2°C) under a bank of fluorescent light (128μ m-2 s-1 at middle of plant height) giving a 14 h photoperiod. These plants were used for various tests in this study.

### Psyllids and Water Stress Test

Three fully developed green leaves were selected on each replicate plant. A 10 cm wide and 18 cm long sleeve made of nylon net was slipped on the branch covering the selected leaves and was firmly fastened to the branch with a string. About fifty psyllids were introduced into the bag and they were allowed to feed on the leaves for five days. At the end of five day interaction (feeding) period, any psyllids clinging to the leaf surfaces were shaken off and the leaves were immediately placed in a -80°C freezer until needed for processing and analysis.

The moisture content in pot-mix (peat: perlite: vermiculite; 1:1:1 by wt.) of six replicate Satsuma plants was reduced to 20% of the water holding and was maintained for 5 days parallel to the psyllids test. The moisture content in the pots of the untreated control plants and the plants used for ACP infestation test was maintained at 80% of water holding capacity throughout the experiment. The individual plants were weighed every day and the moisture content was adjusted by adding small amounts of water to reach 20 or 80% level in stressed and non-stressed plants, respectively. The water adjustments were made by daily measurements of weight of the pots.

### Sampling and Processing

Leaves from psyllid infested, water stressed and control plants were detached at the base and frozen at −80°C until used for biochemical analysis. Frozen leaf samples were pulverized in liquid nitrogen using mortar and pestle (50-500 μ particle size) according to our standard and published method [23, 24]. Pulverized material was used either immediately or stored at −80°C until needed for extraction.

### Polyphenol Extraction

A 60 mg aliquot of powdered Satsuma leaf material, as described above, was added into a 2 mL microfuge tube and was immediately mixed with 1 mL of 80% methanol. The mixture was homogenized for 30 seconds three times at 10 second intervals followed by 10 seconds of sonication (Branson 150I, Fisher Scientific, Danbury, CT) with a Branson 4C15 probe at high power. The homogenized slurry was centrifuged for 30 min at 20,000 rpm at 20ºC using an Eppendorf (5417R, Westbury, NY ) refrigerated centrifuge. After centrifugation the supernatant was decanted into a separate 2 mL microfuge tube and the pellet was re-extracted two more times with 500 μL of 80% methanol under the same conditions. The supernatant liquids from the three extracts were pooled and the pooled extract was then concentrated to dryness with Speedvac (SPD1010, Thermo Savant, Holbrook, NY). The dried samples were reconstituted in 80% methanol to a final volume of 500 μL and filtered through a Costar® Spin-X® filter (0.2μm). The filtrate was then stored at -80ºC until used for analysis by HPLC. Triplicate sample of leaves were extracted for each treatment and each leaf sample was extracted in triplicate and each extract was chromatographed on HPLC in triplicates. The same replication scheme was followed for all biochemical analysis described here.

### High Performance Liquid Chromatography of Polyphenols

Chromatographic separation of phenolic compounds present in methanolic Satsuma leaf extracts was performed by means of a Waters Alliance 2695 HPLC system (Waters, Milford, MA) equipped with a 2996 photo-diode array detector using a Waters Xterra MS C18 column (5μm particle size; 3.9 mm x 150m) maintained at 35°C, and eluted at 1 mL/min as described previously [25]. Phenolic compounds were identified by matching retention times (tr) and the UV spectra in the extract with the peak and the spectra of known standard compounds. For quantitative measurement of dif-

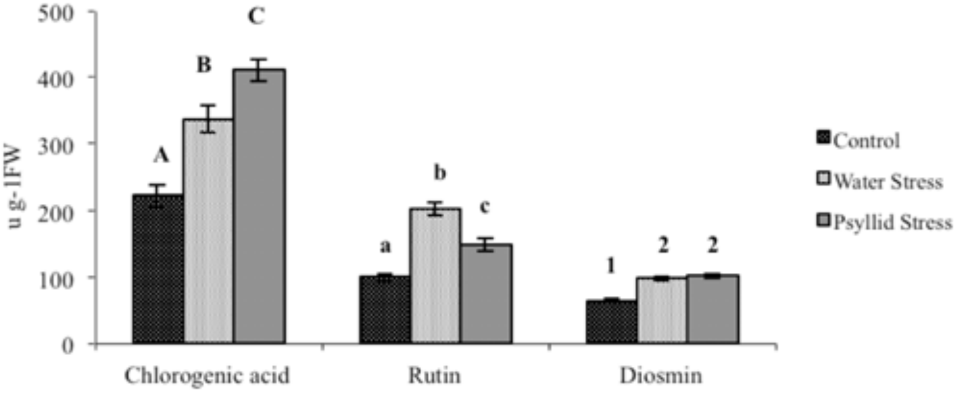

**Fig. (1)**. Changes chlorogenic acid, rutin, and diosmin contents (µ g-1) in Satsuma leaves in response to water and Psyllids stress. Bars indicate standard error (n=9). For each polyphenol. different letters or numbers indicate significant difference ($P < 0.05$).

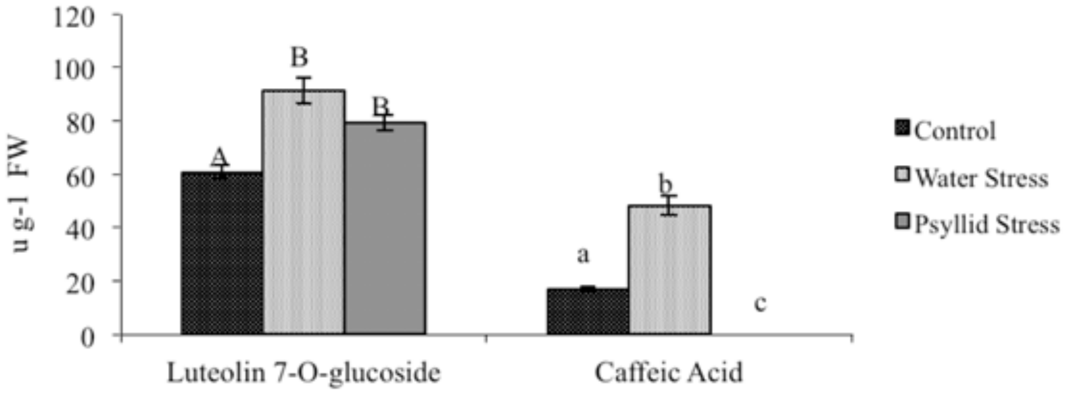

**Fig. (2)**. Changes in luteolin 7-O-glucoside and caffeic acid concentration in response to water and psyllid stress in leaves of Satsuma (Citrus unshiu; cultivar Owari). FW means the fresh weight of the tissue. Different letters indicate significant difference at $P < 0.05$.

ferent polyphenols in the extracts, regression curves were developed using known standard compounds.

## RESULTS

As a general trend, phenolic compounds tended to increase in levels in response to the stress applied to the Satsuma plant. Five out of the eight phenolic compounds identified in this study exhibited increases in their levels in response to stress conditions. The increased levels of chlorogenic acid, rutin, and diosmin in response to both water and insect stress conditions are presented in Fig. (**2**). Endogenous levels of chlorogenic acid in Satsuma leaves increased by 52 and 85% in water and psyllids stressed plants, respectively (Fig. **1**). The levels of rutin also dramatically increased by 103% in water stressed plants as compared to the control while the levels of the same compound in insect stressed plants increased by only 49%; these and other differences reported here were significantly different from controls. A similar trend was found regarding changes in luteolin7-O-glucoside levels. For example, an increase in the level of luteolin 7-O-glucoside by 50 and 31% was observed in water and psyllids stressed Satsuma leaves, respectively (Fig. **2**). The levels of diosmin increased by an average of approximately 50% under both stress conditions. Lastly, caffeic acid levels was observed to significantly increase (184%) in water stressed plants, but in the case of psyllid stressed plants the caffeic acid levels declined drastically and were not detectable when the samples were taken (Fig. **2**).

Levels of Apigenin 7-O-glucoside decreased only in water stressed plants (140%) but not in plants that were subjected to insect feeding, which maintained levels similar to those of the control plants (Table **1**). The levels of hesperidin remained unchanged under water or insect feeding stresses (Table **1**).

## DISCUSSION

The results presented in this study indicate a general trend of increase in levels of polyphenols in response to both biotic and abiotic stresses. These results are in line with the reports that indicate that plants produce a wide range of secondary metabolites such as polyphenols during their normal growth and that these levels often change in response to stresses [26-29]. These responses may be elicited by interactions with microbes, insect pests, the environment, or the farm management practices. On the other hand, insects also possess various chemoreceptors utilized to identify possible or viable food sources, based on their chemical composition

**Table 1. Changes in hesperidin, naringin and apigenin 7-O-glucoside contents (µ g-1) in Satsuma leaves in response to water and Psyllids stress (n=9) and standard error (±). For each polyphenol different superscript letters indicate significant differences (P<0.05).**

| | **Hesperidin (µ g-1FW ± SE)** | **Naringin (µ g-1FW ± SE)** | **Apigenin 7-O-glucoside (µ g-1 FW ± SE)** |
|---|---|---|---|
| Control | 413.29$^{a}$<br>± 25.66 | 4.09$^{a}$<br>± 0.51 | 6.52$^{1}$<br>± 0.71 |
| Water stress | 342.48$^{a}$<br>± 17.42 | 5.02$^{b}$<br>± 0.06 | 2.72$^{2}$<br>± 0.01 |
| Psyllid stress | 408.55$^{a}$<br>± 17.30 | 4.47$^{b}$<br>± 0.26 | 5.14$^{1}$<br>± 0.52 |

[10, 30, 31]. Plants respond to insects by producing secondary metabolites that are unpalatable and in some cases toxic to possible herbivores [32]. Based on these key concepts, it seems feasible to manage insect pest by understanding and managing levels of secondary metabolites such as polyphenolics compounds.

A marked increase in chlorogenic acid, rutin and diosmin in both insect and water stressed plants were observed. It has been previously reported the chlorogenic acid is an important factor in displaying resistance to feeding activities of insects on plants [33]. Therefore, increase in the levels of chlorogenic acid in response to insect stress may be a defensive reaction against the herbivory action of the psyllid. Similarly, rutin has been reported to work as a biopesticidal molecule by exhibiting anti-feeding activities in several insect pests [34]. Regarding diosmin, it is interesting to note that there was an increase in levels of this compound in insect stressed plants but there was no such response in hesperidin levels. Diosmin is the flavone analogue of hesperidin, it is therefore, intriguing to note the influence of one double bond in the C ring may result in a different response to insect stress. In one of our previous studies, the levels of several polyphenolic molecules were evaluated in response to cold stress in sour orange [7]. The results indicate a loss in levels of several polyphenolics compounds (chlorogenic acid, luteolin 7-glucoside, rutin, caffeic acid, apigenin 7-O-glucoside). It was interesting to note in those studies that cold treatment reduced the levels of these polyphenols actually promoted insect feeding; i.e. made plants vulnerable to insect attack. Thus, there appears a relationship between levels of polyphenols in plants (that may be controlled by abiotic and biotic stress) and the plant vulnerability to attacks by different types of insects. A point that may be significant in designing strategies of pest (e.g., ACP) management.

In general, our results indicate increases of several polyphenolic compounds in response to both biotic and abiotic stresses. These responses may be due to the Satsuma trees trying to adapt or react to the stress condition to which they were subjected. We conclude that Satsuma trees may react to psyllid attack by inciting the biosynthesis of polyphenolics compounds in order to deter further insect feeding behavior. Furthermore, the increases in levels of several polyphenolics compounds when Satsuma trees were subjected to water stress may prove to be a valuable and environmentally friendly tool to manage the Asian citrus psyllid in the field by deterring the feeding and hence possible transmission of HLB at appropriate time. Therefore, developing an appropriate irrigation schedule could be an effective management practice to deter feeding of Psyllids.

## CONFLICT OF INTERESTS

The authors confirm that this article content has no conflicts of interest.

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## REFERENCES

[1] Da Graça JV. Citrus greening disease. Annu Rev Phytopath 1991; 29: 109-36.

[2] Halbert SE, Manjunath KL. Asian citrus psyllids (Sternorrhyncha:Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. Fl Entomol 2004; 87: 330-53.

[3] Bove JM. Hauanglongbing: a destructive,newly emerging, century-old disease of citrus. J Plant Path 2006; 88: 7-37.

[4] Jagoueix S, Bové JM, Garnier M. The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. Int J Syst Bacter 1994; 44: 379-86.

[5] Qureshi JA, Stansly PA. Integrated approaches for managing the Asian citrus psyllid Diaphorina citri (Homoptera: Psyllidae) in Florida. Proc Fla State Hort Soc 2007; 120:110-5.

[6] Qureshi JA, Stansly PA. Dormant season foliar sprays of broad-spectrum insecticides: an effective component of Diaphorina citri (Homoptera: Psyllidae) in citrus orchards. Crop Protection 2010; 29: 860-6.

[7] Malik NS, Perez JL, Patt JE, Zibilske LM, Mangan RL. Increased infestation of Asian citrus psyllids on cold treated sour orange seedlings: its possible relation to biochemical changes in leaves. J Food Agric Environ 2012; 10: 424-9.

[8] Bartwal A, Mall R, Lohani P, Guru SK, Arora S. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. J Plant Growth Regul 2013; 32: 216-32.

[9] Fürstenberg-Hägg J, Zagrobelny M, Bak S. Plant defense against insect herbivores. Intl J Mol Sci 2013; 14: 10242-97.

[10] Hout OB, Nachappa P, Tamborindeguy C. The evolutionary strategies of plant defenses have a dynamic impact on adaptations and interactions of vectors and pathogens. Insect Sci 2013; 20: 297-306.

[11] Tariq M, Rossiter J, Wright D, Staley J. Drought alters interactions between root and foliar herbivores. Oecologia 2013; 172:1-10.

[12] Gutbrodt B, Dorn S, Mody K. Drought stress affects constitutive but not induced herbivore resistance in apple plants. Arthropod-Plant Int 2012; 6: 171-9.

[13] Mody K, Eichenberger D, Dorn S. Stress magnitude matters: different intensities of pulsed water stress produce non-monotonic resistance responses of host plants to insect herbivores. Ecol Entomol 2009; 34:133-43.

[14] Kerchev PI, Fenton B, Foyer CH, Hancock RD. Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signalling pathways. Plant Cell Environ 2012; 35: 441-53.

[15] Lattanzio V, Kroon PA, Quideau S, Treutter D. Plant phenolics—secondary metabolites with diverse functions. Recent Adv Polyphenol Res 2009; 1:1-35.

[16] Djoukeng JD, Arbona V, Argamasilla R, Gomez-Cadenas A. Flavonoid Profiling in leaves of citrus genotypes under different environmental situations. J Agric Food Chem 2008; 56: 11087-97.

[17] Ladanyia M, Ladaniya M. Citrus fruit: biol, tech evalu, Academic Press, Access Online via Elsevier. ebooks.com/328556/cit-rus-fruit/ladanyia-milind-ladaniya-milind/

[18] Simmonds MS. Importance of flavonoids in insect–plant interactions: feeding and oviposition. Phytochemistry 2001; 56: 245-52.

[19] Zhou D-S, Wang C-Z Van Loon J. Chemosensory basis of behavioural plasticity in response to deterrent plant chemicals in the larva of the small cabbage white butterfly pieris rapae. J Insect Physiol 2009; 55: 788-92.

[20] Goławska S, Łukasik I, Goławski A, Kapusta I, Janda B. Alfalfa (Medicago sativa L.) apigenin glycosides and their effect on the pea aphid (Acyrthosiphon pisum). Pol J Environ Stud 2010; 19: 913-20.

[21] War AR, Paulraj MG, Hussain B, Buhroo AA, Ignacimuthu S, Sharma HC. Effect of plant secondary metabolites on legume pod borer, Helicoverpa armigera. J Pest Sci 2013; 86: 399-408.

[22] Maserti BE, Del Carratore R, Croce CD, Podda A *et al.* Comparative analysis of proteome changes induced by the two spotted spider mite Tetranychus urticae and methyl jasmonate in citrus leaves. J Plant Physiol 2011; 168: 392-402.

[23] Malik NS, Bradford JM. A simple protein extraction method for proteomic studies on olive leaves. J Food Agric Environ 2005; 3: 246-8.

[24] Yang XB, Malik NS, Perez JL, Liu TX. Impact of potato psyllid (Hemiptera: Triozidae) feeding on free amino acid composition in potato. Insect Sci 2011; 18: 665-79.

[25] Malik N, Perez J. Quantitative assessment of different phenolic compounds in Texas olive oils versus foreign oils. J Agric Sci Tech 2010; 4: 61-7.

[26] Cheruiyot EK, Mumera LM, Ng'etich WK, Hassanali A., Wichira, F. Polyphenols as potential indicators for drought tolerance in tea (Camellia sinensis L.). Biosci Biotech Biochem 2007; 71: 2190-7.

[27] Cheruiyot EK, Mumera LM, Ng'etich WK, Hassanali A, Wichira F, Wanyoko JK. Shoot epicatachin and epigallactechin contents respond to water stress in tea [Camellia sinensis (L.) O. Kuntze]. Biosci Biotech Biochem 2008; 72: 1219-26.

[28] Lattanzio V, Lattanzio VM, Cardinali A. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochem Adv Res 2006; 661: 23-67.

[29] Peterlunger E, Sivilotti P, Colussi V. Water Stressed increased-polyphenolic quality in 'Merlot' grapes. Acta Horticulturae (ISHS), 2005; 689: 293-300 http://www.actahort.org/books/689/6-89_34.htm.

[30] Friend J. Phenolic substances and plant disease. Annu Proc Phytochem Soc 1985; 25: 367-72.

[31] Mendez CDMV, Delgado MAR, Rodriguez EMRR, Romero CD. Content of free phenolic compounds in cultivars of potatoes harvested in Tenerife (Canary Islands). J Agric Food Chem 2004; 52: 1323-7.

[32] Felton GW, Donato K, Del Vecchio RJ. Duffey SS. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J Chem Ecol 1989; 15: 2667-94.

[33] Leiss KA, Maltese F, Choi YH, Verpoorte R, Klinkhamer PG Identification of chlorogenic acid as a resistance factor for thrips in chrysanthemum. Plant Physiol 2009; 150: 1567-75.

[34] Pandey A, Misra P, Chandrashekar K, Trivedi PK. Development of AtMYB12-expressing transgenic tobacco callus culture for production of rutin with biopesticidal potential. Plant Cell Rep 2012; 31: 1867-76.

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