Effects of Exogenous Gibberellic Acid in Huanglongbing-affected Sweet Orange Trees under Florida Conditions – I. Flower Bud Emergence and Flower Formation

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Abstract. Under Florida conditions, sweet orange (*Citrus sinensis*) affected by Huanglongbing {HLB [*Candidatus Liberibacter asiaticus* (CLAs)]} frequently exhibits irregular flowering patterns, including off-season flowering and prolonged bloom period. Such patterns can increase the opportunity for temporal and spatial proliferation of pathogens that infect flower petals, including the fungal causal agent for postbloom fruit drop (PFD) *Colletotrichum acutatum* J.H. Simmonds. For the development of strategies to manipulate flowering, the effects of floral inhibitor gibberellic acid (GA₃) sprayed monthly at full- and half-strength rates (49 and 25 g·ha⁻¹, or 33 and 17 mg·L⁻¹, respectively) with different regimens, starting from September and ending in November, December, or January, on the pattern of spring bloom were evaluated in field-grown HLB-affected ‘Valencia’ sweet orange at two locations in subsequent February through April for two separate years in this study. To further examine whether GA₃ effects on flowering patterns vary in different cultivars, early-maturing ‘Navel’ sweet orange trees receiving no GA₃ or full-strength GA₃ monthly in September through January were included. Overall, for ‘Valencia’ sweet orange, monthly applications of GA₃ at 49 g·ha⁻¹ from September to December not only minimized the incidence of scattered emergence of flower buds and open flowers before the major bloom but also shortened the duration of flowering, compared with the untreated control trees. In addition, exogenous GA₃ led to decreased leaf *flowering locus t* (*FT*) expression starting in December, as well as reduced expression of its downstream flower genes in buds during later months. When applied monthly from September through January at 49 g·ha⁻¹, similar influences of exogenous GA₃ on repressing flower bud formation and compressing bloom period were observed in ‘Navel’ sweet orange. These results suggest that by effectively manipulating flowering in HLB-affected sweet orange trees under the Florida climate conditions, exogenous GA₃ may be used to reduce early sporadic flowering and thereby shorten the window of *C. acutatum* infection that causes loss in fruit production.

In citrus (*Citrus* sp.), the length of bloom period among individual trees in the same block determines the uniformity of maturity of fruit within a grove. In addition, the flowering pattern in citrus trees, including the duration and intensity of seasonal bloom as well as the occurrence of off-season flowering, is closely related to the flower-infecting

diseases that have adverse impacts on fruit production, including PFD. From 2014 to 2016, there were major outbreaks of PFD due to the warm and wet weathers during anthesis in Florida that led to significant yield reduction, with Valencia and Navel being the most susceptible sweet orange cultivars (Dewdney, 2015, 2017; Dewdney and Graham, 2016). Severe PFD outbreak takes place when all three conditions—warm temperatures, moisture, and flowers—are present simultaneously in citrus groves (Dewdney, 2017; Peres et al., 2002, 2005; Perondi et al., 2020). The fungal causal agent of PFD, *C. acutatum*, is dispersed rapidly and broadly by wind-blown rain and reproduces on flower buds and flowers in response to petal leachates under moist conditions (Agostini et al., 1993; Peres et al., 2005; Timmer and Peres, 2015; Timmer et al., 1994). Buds with elongated petals and

open flowers infected by *C. acutatum* develop necrotic lesions on petals accompanied with abscission of a large number of young fruitlets at the end of bloom, leaving calyxes, commonly referred to as “buttons,” on pedicels surviving for the life of the tree branch (Peres et al., 2005; Timmer and Peres, 2015; Timmer et al., 1994). Once the petals are infected with *C. acutatum*, fungicides cannot keep newly set fruitlets from abscising (Dewdney, 2017; Peres et al., 2002, 2005; Perondi et al., 2020). Therefore, knowledge of citrus flowering regulation and approaches to manipulate flowering, including temporal distribution and intensity of bloom, would be key in PFD management.

For HLB-affected citrus trees in Florida, irregular flowering patterns, such as off-season bloom and prolonged flowering in spring, have been frequently observed (Dewdney, 2017; Peres and Dewdney, 2019; Stover et al., 2016). In Florida, intermittent periods of warm temperatures following winter low temperatures that are sufficient for floral induction (20 °C day and 15 °C night temperatures) initiate bud differentiation and thereby lead to multiple waves of bloom, contributing to an extended duration of subsequent flowering (Albrigo, 2004; Valiente and Albrigo, 2002). Furthermore, HLB likely introduces variability in the behavior of flowering by imposing persistent stresses on affected trees. On infection with CLAs, the bacterial agent associated with HLB, trees undergo tremendous dieback of feeder roots (Graham et al., 2013; Johnson et al., 2014), which possibly restricts water uptake from the growing substrate (Hamido et al., 2017). Consistently, Tang et al. (2020) documented lower water potential in ‘Valencia’ sweet orange trees exhibiting severe HLB symptoms compared with mildly symptomatic trees under field conditions, suggesting HLB-affected trees are prone to water-deficit stress in a manner dependent on disease severity. Relevantly, flowering in citrus can be induced by external stimuli, including water-deficit stress (Chica and Albrigo, 2013; Lovatt et al., 1988; Southwick and Davenport, 1986). Limited rainfall during the Florida dry season (from October through May) may further increase the incidence of off-season flowering in HLB-affected trees with a compromised root system (Johnson et al., 2014) in fall and early winter.

To prevent inoculum amplification of pathogens that infect petals of early bloom, including *C. acutatum*, and thereby reduce pesticide usage, strategies to compress and synchronize spring bloom and to eliminate sporadic flowering before the major bloom awaits development. Owing to its inhibitory effect on flowering in citrus (Goldberg-Moeller et al., 2013; Lord and Eckard, 1987; Muñoz-Fambuena et al., 2012), the plant growth regulator GA₃ serves as a good candidate to be used for bloom manipulation. Nevertheless, the current knowledge of GA₃ with respect to citrus flowering has been limited to healthy trees in groves or greenhouses with no presence of HLB reported. The goal of

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Table 1. Application rates (25 and 49 g·ha⁻¹) and regimens (three, four, and five monthly applications) of gibberellic acid (GA₃) treatments to Huanglongbing-affected ‘Valencia’ and ‘Navel’ sweet orange for 3 consecutive years in 2016–17, 2017–18, and 2018–19.

Treatment name	GA ₃ application rate (g·ha ⁻¹)	GA ₃ application regimen	Treated sweet orange cultivar
GA-0	0	None	Valencia and Navel
GA-30	25 ^z	September, October, and November	Valencia
GA-40	25	September, October, November, and December	Valencia
GA-50	25	September, October, November, December, and January	Valencia
GA-60	49	September, October, and November	Valencia
GA-80	49	September, October, November, and December	Valencia
GA-100	49	September, October, November, December, and January	Valencia and Navel

^zApplications of GA₃ at 25 and 49 g·ha⁻¹ (10 and 20 g·ac⁻¹, or ≈33 and 17 mg·L⁻¹, respectively) refer to half- and full-strength rates, respectively, in this study.

this research was therefore to shed light on the influences of exogenous GA₃ on flowering in citrus trees affected by HLB.

The first objective of this study was to determine the timing and rate of GA₃ treatments that effectively reduce and shorten citrus bloom under Florida conditions. Hence, foliar sprays of GA₃ at two rates (25 and 49 g·ha⁻¹, or 33 and 17 mg·L⁻¹, respectively) were made monthly starting in September, before the low temperatures that trigger floral induction (Krajewski and Rabe, 1995; Lord and Eckard, 1985, 1987; Nishikawa et al., 2009), and ended in November, December, or January (for a total of three, four, or five applications). Notably, all application regimens also overlapped the period of fall through early winter, during which off-season bloom usually happens (L. Tang and T. Vashisth, unpublished data). A subsidiary aim under the first research objective was to gain an understanding of the dynamic of endogenous GAs in relation to exogenous GA₃ and flower development; thus, the concentration of GAs and relative expression of floral genes were analyzed in buds of ‘Valencia’ sweet orange trees with two extreme application regimens, that is, untreated control and applications of GA₃ at 49 g·ha⁻¹ from September through January. Because fruit (seeds), a source of endogenous GA₃, act as a strong inhibitor of flowering (Iglesias et al., 2007; Monselise and Goldschmidt, 1982; Plummer et al., 1989; Talón et al., 1990), the second objective was to compare two sweet orange cultivars with different maturity times—early-maturing Navel sweet orange harvested in November and late-maturing Valencia sweet orange harvested in April to May—in relation to the effects of exogenously supplied GA₃. Although flowering in response to exogenous GA₃ was the focus of the present report, the effects of GA₃ treatments on fruit production as well as overall tree health in HLB-affected sweet orange trees were evaluated and are discussed in the second report by our group titled “Effects of Exogenous Gibberellic Acid in Huanglongbing-Affected Sweet Orange Trees under Florida Conditions – II. Fruit Production and Tree Health” (S. Singh, T. Livingston, L. Tang, and T. Vashisth, unpublished data).

Materials and Methods

Plant materials and treatment conditions. Eight and 10-year-old ‘Valencia’ sweet orange on ‘Swingle’ citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) rootstock and 18-

and 15-year-old ‘Navel’ sweet orange on ‘Swingle’ citrumelo rootstock in commercial groves in Fort Meade (FM) and Haines City (HC), which are located on the central ridge Florida, were used. At each site, trees that were relatively uniform in height and canopy volume were selected. Due to the prevalence of HLB in Florida, citrus trees under open-air field conditions are rarely CLas-negative. All sweet orange trees in this research also exhibited HLB symptoms, including blotchy mottled leaves and branch dieback, indicating the state of being affected by the disease.

For ‘Valencia’ sweet orange, GA₃ treatments were applied to the same trees for 3 consecutive years (2016–17, 2017–18, and 2018–19). The field experiment in FM and HC was laid out as a randomized complete block design. In each grove, there were four blocks (replications), each containing one untreated control and six GA₃ treatments. Treatments included two rates of exogenous GA₃, based on the existing literature on GA₃ use in flowering research (reviewed by Garmendia et al., 2019), at three application regimens as follows: monthly foliar spray of GA₃ at 20 g·ac⁻¹ (49 g·ha⁻¹ or ≈33 mg·L⁻¹; “full-strength”) from September to November (GA-60), from September to December (GA-80), and from September to January (GA-100); at 10 g·ac⁻¹ (25 g·ha⁻¹ or ≈17 mg·L⁻¹; “half-strength”) from September to November (GA-30), from September to December (GA-40), and from September to January (GA-50) (Table 1). The numbers in treatment names were assigned according to the combination (multiplication) of GA₃ application rate (20 or 10 g·ac⁻¹) and total application times (three, four, or five applications) for easy adoption by citrus growers. For the application, GA₃ (ProGibb LV Plus; Valent BioSciences, Libertyville, IL) mixed with 0.05% (v/v) nonionic surfactant (Induce; Helena Chemical, Collierville, TN) was used. The control (GA-0) trees were not treated with GA₃ but sprayed with an equal amount of water containing surfactant as the GA₃ treatments. All foliar applications were uniformly distributed to the canopy to the point of runoff (≈4.7 L per tree) using a skid sprayer (Chemical Containers, Lake Wales, FL). Three consecutive trees were considered as one experimental unit but only the ones located in the center (one tree) in each unit were used for data and sample collection to allow sufficient buffer space between treatments.

The experiment was implemented for only 1 year (2018–19) for ‘Navel’ sweet orange

trees in FM and HC groves with the same experimental design as for ‘Valencia’ sweet orange. However, only the GA-100 treatment was applied besides untreated control for ‘Navel’ sweet orange because of the limited number of trees with a similar level of HLB severity, based on the visual assessment, in the groves. Supplemental Figs. 1 and 2 depict the specific dates of GA₃ applications for ‘Valencia’ sweet orange in 3 years (2016–17, 2017–18, 2018–19) and ‘Navel’ sweet orange trees in 2018–19. Monthly average maximum and minimum air temperatures for 2017–18 and 2018–19 (Supplemental Fig. 3) were downloaded from the Florida Automated Weather Station Network for the closest station to each grove.

Evaluation of flower bud emergence, bloom, and off-season flowering. The quantity of flower buds and open flowers was assessed in all treatments of the two cultivars and the two sites every 2 weeks from January through April (full bloom) using the method described by Cooper et al. (1963) and Hall and Albrigo (2007) with some modifications. Elongated flower buds [at the popcorn stage with elongated petals (Dewdney et al., 2019)] and open flowers were counted within a cubic square frame (0.25 × 0.25 × 0.25 m, 15.63 dm³), made of polyvinylchloride pipes, placed onto the outer tree canopy at the height of ≈1.5 m above the ground. For each tree, the results recorded from two frames on the east and west side of the canopy (two frames/tree) were averaged and reported herein. The number of total flower buds produced over the course of spring was calculated by adding up all flower bud numbers on individual survey days in each tree; the sum of flower buds was further used to calculate the cumulative sprouting rate of flower buds on each sampling day.

In the first year of the study (2016–17), ‘Valencia’ sweet orange trees at both sites were hedged in early March of 2017 by the growers, leading to the difficulty in accurately quantifying flower buds and open flowers thereafter even though those parameters were still recorded (Supplemental Fig. 4). Thus, to avoid any invalidity, the data collected in 2016–17 were not included in the analysis and discussion in the current study.

Young developing fruit (with transverse diameter <4.0 cm) were recorded using the cubic square frame method mentioned previously from January to February for ‘Valencia’ sweet orange and in December to

February for 'Navel' sweet orange to represent the occurrence of off-season flowering that might have taken place in late fall or early winter. In addition, to assess whether PFD was affected by the GA₃ applications, calyxes or buttons (where *C. acutatum*-caused fruitlet abscission takes place) were manually quantified on all trees after initial fruit set in subsequent June at both sites for two cultivars.

Sample collection and gene expression analysis. For 'Valencia' sweet orange located in FM in the 2018–19 experiment, 15 shoots with leaves were collected randomly from trees of the two extreme treatments—untreated control (GA-0) and GA-100—on 13 Dec. 2018, and 11 Jan., 7 Feb., 18 Feb., 5 Mar., and 20 Mar. 2019 (*n* = 4). It should be noted that the samplings in December and January were done before the GA₃ application in the corresponding months. After collection, the shoot tissues were quickly transferred to the laboratory on ice. Subsequently, leaves and buds were excised from the shoot, immediately frozen in liquid nitrogen, and stored at –80 °C in the freezer until further use.

Total RNA was extracted from 100 mg of the leaf and bud tissue with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). After quality and quantity were validated using a spectrophotometer (Epoch 2 Microplate; BioTek Instruments, Winooski, VT) and denaturing formaldehyde 1.2% agarose gels (Rio, 2015), 1 µg RNA was used for complementary DNA synthesis with DNase I (Promega, Madison, WI), oligo (dT)₁₅ primer, dNTP mix, and reverse transcriptase (ImProm-II; Promega) according to the manufacturers' protocols. Subsequently, expression levels of *FT*, *apetala1* (*API*), *leafy* (*LFY*), and *suppressor of overexpression of constans1* (*SOC1*), selected based on their roles in citrus flower development (Goldberg-Moeller et al., 2013; Sousa et al., 2019) (primer information listed in Supplemental Table 1), were determined using a real-time quantitative polymerase chain reaction (qPCR) system (7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA). The qPCR reactions with dissociation-curve analysis, which confirmed that no nonspecific products were formed, were carried out following the procedures described by Tang and Vashisth (2020). Using the quantification cycle (Ct), the levels of relative expression of genes of interest were expressed by the normalized relative quantity (NRQ) calculated with two reference genes, *actin7* and *dim1* (Mafra et al., 2012, 2013), as internal control using the Pfaffl method (Hellems et al., 2007; Pfaffl, 2001; Rieu and Powers, 2009) for visual presentation. For statistical analysis, log₂ NRQ was used to account for heterogeneity of variance of the NRQ data based on Rieu and Powers (2009).

Analysis of endogenous gibberellic acids. For GA quantification, 100 mg (fresh weight) of frozen bud tissues collected from 'Valencia' sweet orange trees in FM on 11 Dec. 2018, and 11 Jan. and 2 Feb. 2019 were sent to the Proteomics and Metabolomics Facility, Nebraska Center for Biotechnology,

University of Nebraska-Lincoln. Gibberellic acids, including GA₁, GA₃, GA₄, GA₈, GA₉, GA₁₂, GA₁₉, GA₂₀, GA₂₄, GA₂₉, and GA₅₃, were extracted and analyzed using liquid chromatography–mass spectrometry targeted assay as described by Hung et al. (2016).

Statistical analysis. Statistical analyses were performed using R version 3.6.2 (R Core Team, 2020) and SigmaPlot version 13 (Systat Software, San Jose, CA). Repeated-measures analysis of variance was used to determine the treatment effects on the number of total elongated flower buds and sprouting rate of flower buds (after arcsine square root transformation of the percentage data), followed by the Holm-Sidak method (at *P* = 0.05) for post hoc mean separation at individual time points. Within the treatment, the number of open flowers on different days was compared using Dunnett's test, with first survey day as control; significant differences compared with the first day were noted as "pronounced flowering" dates in this study. Student's *t* test was used to test for the difference in gene expression levels and GA₃ concentrations in the GA-0 and GA-100 'Valencia' sweet orange trees as well as the effects of GA-100 treatments on flowering parameters in 'Navel' sweet orange.

Results

Flower bud sprouting in 'Valencia' sweet orange. In 2018, the untreated control 'Valencia' sweet orange trees (GA-0) located in FM produced 234 (unopen) elongated flower buds per frame (0.25 × 0.25 × 0.25 m, 15.63 dm³) in total in the spring (Fig. 1A). For the GA-0 trees, a substantial portion of flower buds (39% of total) emerged on 12 Feb. (Fig. 1B, Supplemental Fig. 5A). Trees applied with full-strength GA₃ monthly starting in September to November (GA-60), to December (GA-80), and to January (GA-100) had 71, 62, and 65 flower buds per frame (≤30% of the GA-0 trees), respectively, over the course of the experiment, significantly fewer than that of the GA-0 trees (Fig. 1A). When the concentration of GA₃ was lowered by half, only the applications made in September to January (GA-50) resulted in a significant decrease in total flower buds per frame compared with the untreated control (Fig. 1A).

In 2019, for 'Valencia' sweet orange at the FM site, before most buds emerged on 7 Mar. (88% of total flower buds), a small quantity of flower buds sporadically sprouted on 5 and 18 Feb. (3% and 18% of total flower buds, respectively) in GA-0 trees (Fig. 1D and Supplemental Fig. 5B), in contrast to the previous year. Despite lacking the significant reduction in the sum of flower buds in spring of 2019 (Fig. 1C), all GA₃ treatments were able to hold off the bud emergence rate to less than 2% in February before the major sprouting in early March (Fig. 1D).

For 'Valencia' sweet orange grown in the HC grove, the three full-strength GA₃ treatments significantly reduced the number of total flower buds per frame compared with

the untreated control in 2018 (Fig. 1E), whereas monthly GA₃ applications were only effective when made through January at either rate (GA-50 and GA-100) in 2019 (Fig. 1G). In both years, the GA-0 trees exhibited a pattern of sporadic bud sprouting in early spring (21% of total flower buds on 13 Feb. 2018 and 7% of total on 4 Feb. 2019) before the emergence of most buds in late February (Fig. 1F and H). In 2018, the sprouting rate of flower buds on 13 Feb. dropped to less than 3% in response to all GA₃ treatments, significantly lower than that of the untreated control (Fig. 1F). In the following year, early bud emergence (on 4 Feb. 2019) was also brought down by all treatments, except GA-50, to merely 1% to 4% before the major sprouting (Fig. 1H), even though the difference was not statistically significant.

Temporal distribution and intensity of bloom in 'Valencia' sweet orange. In 2018, the first instance of open flowers was observed on 21 Feb. (eight flowers/frame) followed by the maximum bloom on 6 Mar. (23 flowers/frame) in the GA-0 'Valencia' sweet orange trees in FM (Fig. 2A); on both days, the number of open flowers was significantly greater than that of the first survey day (30 Jan.). Although flowering also occurred in the GA-0 trees on 21 Mar., the very few open flowers (two flowers/frame) was not different from the number on 30 Jan. Together, the results indicated that in the absence of exogenous GA, there were two dates of "pronounced flowering" for trees located in FM in 2018. In the spring following the treatments in 2018–19, the GA-0 trees also had two dates of pronounced flowering on 7 and 18 Mar. 2019 (Fig. 2B).

Two weeks before the maximum bloom in 2018 and 2019, the number of open flowers was decreased, either numerically or significantly, by all full-strength GA₃ treatments (<1 and <5 flowers/frame on 21 Feb. 2018 and 7 Mar. 2019, respectively) compared with the GA-0 trees (Fig. 2A and B). As a result, the GA-60 and GA-80 trees had only one date of pronounced flowering. Nevertheless, in either year, such a pattern of condensed bloom period was not observed in the GA-100 trees, which had two dates of pronounced flowering—on 6 and 21 Mar. 2018 and 18 Mar. and 2 Apr. 2019—demonstrating a delay in bloom. For the half-strength application regimens, both GA-40 and GA-50 reduced pronounced flowering to one date over the season in the FM grove for 2 continuous years (Fig. 2A and B).

For 'Valencia' sweet orange in HC, the untreated control trees had only one date of pronounced flowering for 2 years in a row (Fig. 2C and D), in contrast to the extended spring bloom observed in trees in FM. Therefore, the effect of GA₃ treatments on eliminating sporadic flowering before the peak bloom or on compressing the duration of bloom was unnoticeable in trees located in HC. It is noteworthy that, albeit not significantly different, the GA-80 and GA-40 trees had fewer open flowers (fewer than one

Fig. 1. Number of total elongated flower buds per frame produced in spring and cumulative rate of flower buds on individual survey days in untreated control (GA-0) 'Valencia' sweet orange trees and trees applied with gibberellic acid monthly from September to November, September to December, and September to January at the rate of 25 (GA-30, GA-40, and GA-50, respectively) and 49 g·ha⁻¹ (GA-60, GA-80, and GA-100, respectively) in Fort Meade and Haines City in 2018 (A, B, E, F) and 2019 (C, D, G, H). Data are means $\pm$ SD of four biological replicates. Within a column (day), means followed by the same letters are not significantly different ($P < 0.05$). NS = not significantly different.

flower/frame) than the GA-0 trees (eight flowers/frame) on 20 Feb. 2019, 2 weeks earlier than the maximum flowering.

Floral gene expression in buds and leaves of 'Valencia' sweet orange. Compared with the untreated control, the GA-100 treatments resulted in a decrease in floral intensity (the sum of flower buds and open flowers) on 5 Feb., 18 Feb., and 7 Mar. 2019 in 'Valencia' sweet orange trees grown in FM (Fig. 3A). After receiving GA₃ sprays in September,

October, and November, the GA-100 trees had significantly lower transcript levels of *FT* in leaves and *FT*, *LFY*, and *API* in buds than those of the GA-0 trees on 13 Dec. 2018, 2 months before the first significant decrease in floral intensity on 5 Feb. 2019 (Fig. 3B). The GA₃ treatment continued to result in a significant reduction in *FT* expression on 11 Jan. and 7 Feb. 2019, *API* expression on 11 Jan. 2019, and *LFY* expression on 18 Feb. 2019 in buds compared with the untreated control.

There was no significant difference in bud *SOC1* expression between the GA-100 and GA-0 trees on any sampling day.

Endogenous gibberellic acid concentrations in buds of 'Valencia' sweet orange. Gibberellin acids in buds collected on 13 Dec. 2018, 11 Jan. 2019, and 2 Feb. 2019 from the GA-0 and GA-100 'Valencia' sweet trees grown in FM were analyzed. Across all sampling days, the concentrations of GA₁, GA₃, GA₄, GA₈, GA₁₂, GA₁₉, GA₂₀, GA₂₄, and GA₅₃

Fig. 2. Number of open flowers per frame in untreated control (GA-0) 'Valencia' sweet orange trees and trees applied with gibberellic acid monthly from September to November, September to December, and September to January at the rate of 25 (GA-30, GA-40, and GA-50, respectively) and 49 g·ha⁻¹ (GA-60, GA-80, and GA-100, respectively) in Fort Meade in 2018 (A) and 2019 (B) and Haines City in 2018 (C) and 2019 (D). Data are means $\pm$ SD of (continued) four biological replicates. Within each treatment, asterisks indicate significant difference compared with the number on the first survey day based on Dunnett's test. Within a column (day), means followed by the same lower or upper case letters are not significantly different ($P < 0.05$). Different letters indicate significant differences ($P < 0.05$) among treatments on the individual survey days.

Fig. 3. Floral intensity, expressed as the sum of elongated flower buds and open flowers per frame, and the relative expression of *flowering locus t* (*FT*), *suppressor of overexpression of constans1* (*SOC1*), *leafy* (*LFY*), and *apetalal* (*AP1*) in untreated control (GA-0) ‘Valencia’ sweet orange trees and trees applied with 49 g·ha⁻¹ gibberellic acid monthly from September to January (GA-100). Asterisks indicate significant differences between two treatments on individual sampling days.

were below the limit of detection in buds of the GA-0 and GA-100 trees (Table 2). Whereas GA₉ was detected in buds of both treatments on all collection days, GA₂₉ was detected only on 13 Dec. 2018 (Table 2). Nevertheless, there was no significant difference in the level of detected GA₉ between the GA-0 and GA-100 treatments on any day. On the other hand, bud GA₂₉ concentration in the GA-100 trees was significantly lower than that in the untreated control trees on 13 Dec. 2018, indicating that three monthly applications (made in September, October, and November) of full-strength GA₃ might bring about a decrease in GA₂₉ in the bud. In plants, GA₂₉ could potentially compete with bioactive GA₁ and inactive GA₅, which can turn into either bioactive GA₃ or inactive GA₆, for the substrate GA₂₀ (Sun, 2008). Therefore, whether bioactive GAs can be upregulated endogenously in citrus buds in response to exogenously applied GA₃ remains inconclusive.

Flower bud sprouting in ‘Navel’ sweet orange. For ‘Navel’ sweet orange in FM, the GA-100 trees had 128 elongated flower buds

cumulatively counted per frame over the course of spring in 2019, which was one-third fewer than the number of flower buds produced by the untreated control (GA-0) trees (192 flower buds), although not significantly ($P = 0.07$) (Fig. 4A). During the same year in HC, the GA-100 treatment uniformly resulted in a significant decrease in total flower buds to 92 in spring, in comparison with 227 flower buds produced by the GA-0 trees (Fig. 4C).

In 2019, the GA-0 trees of ‘Navel’ sweet orange at FM and HC sites exhibited a pattern of scattered bud sprouting early in the season before a large population emerged on 5 Feb. (28% of total flower buds; Fig. 4B) and 4 Feb. (36% of total flower buds; Fig. 4D), respectively. To be specific, 5% and 11% of total flower buds emerged on 4 and 23 Jan., respectively, in the GA-0 trees in FM (Fig. 4B); for the untreated control trees in HC, 3% of total flower buds were observed on 10 Jan. and 12% on 22 Jan. (Fig. 4D). In parallel to the effect of GA₃ treatments in ‘Valencia’ sweet

orange, exogenous GA₃ constantly reduced early bud sprouting to 2% on 4 Jan. and 4% on 23 Jan. in ‘Navel’ sweet orange trees in FM (Fig. 4B) and to less than 3% on 10 and 22 Jan. for trees in the HC grove (Fig. 4D).

Temporal distribution and intensity of bloom in ‘Navel’ sweet orange. For the GA-0 ‘Navel’ sweet orange trees in FM, a small quantity of open flowers (fewer than five flowers/frame) were observed on 4 Jan., 23 Jan., and 5 Feb., followed by a substantial number of flowers on 19 Feb. (12 flowers/frame), significantly greater than that of the first survey day in December of the previous year (Fig. 5A). On 8 and 18 Mar., the number of open flowers remained remarkably greater than the first day, leading to a total of three dates of pronounced flowering in spring for the untreated control ‘Navel’ sweet orange trees. Monthly applications of full-strength GA₃ from September to January effectively reduced the number of open flowers on 5 Feb., 19 Feb., and 8 Mar. (fewer than five flowers/frame) compared with the GA-0

Table 2. Concentrations of gibberellic acids (GAs) in buds of untreated control (GA-0) 'Valencia' sweet orange trees and trees applied with 49 g·ha⁻¹ GA monthly from September to January (GA-100).

Date	Treatment	GA ^z concn (ng·g ⁻¹)			
		GA ₉	<i>P</i> value ^y	GA ₂₉	<i>P</i> value
13 Dec. 2018	GA-0	1109 ^x	0.647	209	0.001
	GA-100	1279		136	
11 Jan. 2019	GA-0	879	0.051	ND	na
	GA-100	1376		ND	
2 Feb. 2019	GA-0	686	0.282	ND	na
	GA-100	842		ND	

^zThe levels of GA₁, GA₃, GA₄, GA₈, GA₁₂, GA₁₉, GA₂₀, GA₂₄, and GA₅₃ were below the detection limit and therefore not presented in this table.

^yTreatment effects on individual days are determined using Student's *t* test.

^xData are means of four biological replicates; ND = not detected; na = not available.

trees, albeit not statistically significant on 8 Mar., leaving only one date of pronounced flowering for the GA-100 trees of 'Navel' sweet orange on 18 Mar. (Fig. 5A). Similarly, the GA-100 trees in HC produced significantly fewer open flowers on 20 Feb. and 4 Mar. compared with the untreated control, and had one sole date of pronounced flowering in spring (Fig. 5B).

Off-season bloom 'Valencia' and 'Navel' sweet orange. In this research, young developing fruit (with transverse diameter <4.0 cm) were quantified up until early February to represent the occurrence of off-season flowering that might take place in late fall or early winter in Florida. For 'Valencia' sweet orange, zero fruit was recorded during the

period of January through February for the GA-0 trees, and those subjected to any of the treatments grown at both sites across years. In contrast, a small number of developing fruit (fewer than eight fruit/frame) was observed in the GA-0 trees of 'Navel' sweet orange in FM and HC in the beginning of January (Fig. 6). The fruit number in the GA-100 trees, nonetheless, was not significantly less than that of the control on any sampling day, indicating the incidence of off-season flowering might not be vastly different between the two.

PFD in 'Valencia' and 'Navel' sweet orange. For sweet orange trees of all treatments (including non-GA₃-treated control) in this research, the number of buttons per

frame was always less than 10 in June (after initial fruit set) 2017, 2018, and 2019 (data not shown), indicating PFD outbreak did not occur in either grove when the current study was conducted. It should be noted that there was also no report of severe PFD outbreak during those years (Summer 2017–19) for other locations in Florida.

Discussion

The results reported herein demonstrate distinctions in the flowering behavior between HLB-affected 'Valencia' and 'Navel' sweet orange under field conditions. In the absence of exogenous GA, the trend of off-season bloom, estimated using the number of developing fruit (zero fruit for all cases), was not apparent in 'Valencia' sweet orange trees at either site for 2 consecutive years. In contrast, the observation of small fruit on non-GA-treated 'Navel' sweet orange trees as early as January indicated that a few flowers were produced in November or early December the previous year (Fig. 6). The obvious pattern of off-season flowering in 'Navel' sweet orange may be attributed to the relatively short duration of fruit present on trees. In this study, Navel, an early-maturing cultivar, was harvested in November, which coincided with the estimated time of off-season bloom, whereas harvests of late-maturing 'Valencia' sweet orange did not happen until the following April to May (Supplemental Figs. 1 and 2).

Fig. 4. Number of total elongated flower buds per frame produced in spring and cumulative rate of flower buds on individual survey days in untreated control (GA-0) 'Navel' sweet orange trees and trees applied with gibberellic acid monthly from September to January at the rate of 49 g·ha⁻¹ (GA-100) in Fort Meade (A, B) and Haines City (C, D). Data are means ± SD of four biological replicates. Asterisks indicate significant differences between two treatments. NS = not significantly different.

Fig. 5. Number of open flowers per frame in untreated control (GA-0) 'Navel' sweet orange trees and trees applied with gibberellic acid monthly from September to January at the rate of 49 g·ha⁻¹ (GA-100) in Fort Meade (A) and Haines City (B). Within each treatment, asterisks indicate significant difference compared with the number on the first survey day based on Dunnett's test. Daggers and double daggers indicate significant differences of two treatments at $P < 0.05$ and 0.01, respectively.

It is relevant that fruit collectively act as a strong inhibitor of flower development in citrus (Garcia-Luis et al., 1986; Martínez-Fuentes et al., 2010; Monselise and Goldschmidt, 1982; Shalom et al., 2012). In addition, limited rainfall during the Florida dry season in fall likely subjects HLB-affected citrus trees with already compromised root systems to water-deficit stress (Hamido et al., 2017; Johnson et al., 2014), which triggers flowering (Chica and Albrigo, 2013; Lovatt et al., 1988; Southwick and Davenport, 1986), especially when the floral inhibitor (fruit) is not present, leading to off-season bloom. 'Navel' sweet orange also had flower buds sprouting earlier (in January) (Fig. 4) with more extended period of flowering the following spring (two to three dates of pronounced flowering) (Fig. 5) compared with 'Valencia' sweet orange, for which the emergence of elongated buds began in February and there were only one or two dates of pronounced flowering (Figs. 1 and 2). These observations suggest that the inherent factors within cultivars that regulate fruit maturity, which affects the harvest time, play an essential role on phenology of return flowering in citrus trees.

Monthly application of full-strength GA₃ (49 g·ha⁻¹) starting at September through January had no noticeable effect on eliminating off-season flowering occurring in November or December for 'Navel' sweet orange at either

site (Fig. 6), suggesting that the combined influence of fruit removal (harvest) and ambient floral-inductive conditions on promoting flowering negated the inhibition exerted by the exogenous GA₃ sprays. Therefore, to minimize the presence of off-season bloom in 'Navel' sweet orange or early-maturing cultivars, the efficacy of more frequent GA₃ applications and/or at a higher rate on incidental flowering in fall and early winter awaits evaluation.

On the other hand, even though zero developing fruit was present on the canopy of 'Valencia' sweet orange trees from January to February in the current study, this result does not preclude the possible occurrence of flowers produced before the evaluation day. Instead, this may reflect the erratic nature of off-season bloom for field-grown HLB-affected citrus trees in Florida because the behavior of scattered flowering during late fall or early winter has been commonly observed in this cultivar (L. Tang and T. Vashisth, unpublished data).

With the exception of trees in FM in 2018 (Fig. 1B), all untreated control trees of 'Valencia' sweet orange had a small percentage of elongated flower buds growing out before the major sprouting (Fig. 1D, F, and H). The results reported herein demonstrated that such a pattern of sporadic flower bud spouting can be markedly reduced in response to any GA₃ treatments excluding GA-50 treatment, which

resulted in a greater sprouting rate than the control in HC on 2 Feb. 2019. Interestingly, GA-60, GA-80, and GA-100 trees produced a similar amount of total flower buds in each year and location (Fig. 1A, C, E, and G), despite receiving different numbers of GA₃ applications. The results suggest that the effect of exogenous GA₃ is not additive, with extra applications carried out after December. In sweet orange, exogenous GA₃ inhibits flowering by promoting the vegetative development of shoot apical meristems when applied before buds become determined, or irreversibly committed to flower development (Lord and Eckard, 1987). Lord and Eckard (1987) also documented that after the initiation of the first sepal in the terminal meristem in early January, exogenously applied GA₃ had no influence on suppressing citrus flower formation anymore. Undoubtedly, the time of bud determinacy—a meristem development stage after which exogenous GA₃ no longer inhibits citrus flowering—may vary in trees of different studies due to the differences in climatic conditions, especially air temperatures and occurrence of drought stress (Chica and Albrigo, 2013; Tang and Lovatt, 2019). Nevertheless, the results of recent studies demonstrated that in the subtropics of the Northern Hemisphere, applying GA₃ once in early December or four times from mid-November through mid-December (at 2-

Fig. 6. Number of developing fruit (with transverse diameter <4.0 cm) per frame in untreated control (GA-0) 'Navel' sweet orange trees and trees applied with gibberellic acid monthly from September to January at the rate of 49 g·ha⁻¹ (GA-100) in Fort Meade (A) and Haines City (B). Data are means ± SD of four biological replicates. NS = not significantly different.

week intervals) was sufficient to significantly reduce flower intensity at full bloom in healthy field-grown citrus trees (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012). Taken together, the observations of the current research suggest that the effect of exogenous GA₃ on flower suppression may become negligible when the monthly applications were made through January under Florida conditions in comparison with the regimen ended by November or December. This inference is in agreement with the results of gene expression analysis in the GA-100 and GA-0 trees of 'Valencia' sweet orange. Compared with the untreated control, downregulation of *FT* transcripts in leaves and *FT*, *API*, and *LFY* expression in buds of the GA-100 trees took place as early as 13 Dec. 2018, following three monthly applications of GA₃ at the full strength from September to November (Fig. 3B). In citrus, *FT*, *API* and *LFY* act as the positive regulator in citrus flower development (Endo et al., 2005; Pillitteri et al., 2004), analogous to their homologs in *Arabidopsis thaliana* (Bowman et al., 1991; Corbesier et al., 2007; Kinoshita and Richter, 2020; Weigel et al., 1992). Further, their expression levels were strongly correlated with the number of inflorescences in field-grown healthy citrus trees in response to inductive conditions, such as fruit removal and seasonal low temperatures (Nishikawa et al., 2009; Shalom et al., 2014), as well as floral inhibitor GA₃ (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012). In this study, the expression of *SOCI* in buds was not responsive to exogenous GA₃ on any collection day, similar to the results documented by Goldberg-Moeller et al. (2013) and Muñoz-Fambuena et al. (2012), providing evidence that the GA₃-induced downregulation of citrus flower development may not be mediated via the transcription of *SOCI*.

Regardless of the application regimen, the effect of the GA₃ treatments at the full strength on repressing 'Valencia' flower bud formation, represented by the total number of flower buds produced in spring, was more prominent compared with their half-strength (25 g·ha⁻¹) counterparts at both sites in 2018 (Fig. 1A and E), although the trend was inconsistent the following year (Fig. 1C and G). The discrepancy between the years might be due to the slightly greater maximum temperatures during January and February in 2019 than 2018 (Supplemental Fig. 3), which favored the development of flower buds (Albrigo, 2004; Valiente and Albrigo, 2002) and thus prevailed over the inhibitory effect exerted by exogenous GA₃ to some degree.

For 'Valencia' sweet orange, the GA-80 treatment consistently reduced the number of open flowers before the major bloom, minimizing sporadic flowering early in the season. In addition, the duration of spring bloom, indicated by the number of dates of pronounced flowering compared with the first survey day, was shortened in the GA-80 trees compared with the GA-0 trees for 2 consecutive years in FM (Fig. 2A and B). Although such a treatment effect on reducing bloom period was not observed for the GA-80 trees

in the HC grove in either year (Fig. 2C and D), it is notable that the duration of spring flowering for the untreated control trees in HC was not as extended (with only one pronounced flowering date) as for those in FM (having two dates of pronounced flowering). Thus, it is possible that the responses to exogenous GA₃ might be masked by the relatively brief period of seasonal bloom in 'Valencia' sweet orange trees in HC. The results also indicated that despite being the same cultivar and under presumably similar climatic conditions (Supplemental Fig. 3), given the close proximity between the FM and HC groves (<50 km apart), the duration of flowering differs for citrus trees affected by HLB.

For 'Navel' sweet orange, the sole GA₃ treatment in this study, GA-100, resulted in a decrease in the emergence of flower buds and open flowers before the peak bloom and thereby compressed the bloom period in spring, analogous to the effects of the GA-80 treatment in 'Valencia' sweet orange. Nevertheless, whether a regimen with fewer GA₃ applications, GA-80 for example, would cause similar results awaits determination for this early-maturing cultivar.

By reducing the duration and intensity of bloom, GA-80 and GA-100 treatments might be used to limit the buildup of pathogens that infect flowers in 'Valencia' and 'Navel' sweet orange, respectively, under Florida conditions. In the case of PFD caused by *C. acutatum*, outbreak did not occur in either grove when this study was conducted. It should be noted that severe outbreak of PFD takes place only when the environmental conditions in spring are favorable for fungal distribution (i.e., warm temperatures with wetness in the presence of flowers) (Dewdney, 2017; Peres et al., 2002, 2005; Perondi et al., 2020). Thus, as the results reported herein do not indicate or negate the efficacy of GA₃ in PFD control, the effect of altered flowering patterns by exogenous GA₃ on *C. acutatum* proliferation needs to be further examined. Moreover, flowering manipulation with GA₃ may bring supplemental advantage to the vector control of HLB. At present, most insecticides targeting asian citrus psyllid (*Diaphorina citri*), the insect vector that transmits the pathogen that causes HLB (Halbert et al., 2000; Jagoueix et al., 1994), are also toxic to bees, so their uses are not recommended when citrus trees are flowering (Stansly et al., 2019). Hence, the absence of scattered and prolonged flowering ahead of full bloom in GA-treated trees might allow an extended application regimen of insecticides to diminish psyllid population for a more extensive HLB control compared with trees with an irregular flowering pattern.

Conclusions

This research, for the first time, documented the seasonal flowering pattern (i.e., emergence of flower buds and open flowers from January through April), with a quantitative approach in two sweet orange cultivars affected by HLB. The results reported herein

further demonstrated that monthly applications of full-strength GA₃ (49 g·ha⁻¹) from September through December (GA-80) and from September to January (GA-100) are the most effective and consistent treatments to manipulate spring flowering in HLB-affected 'Valencia' and 'Navel' sweet orange, respectively. Specifically, the two GA₃ treatments minimized the presence of elongated buds and open flowers before the peak bloom and shortened the length of spring flowering. Thus, with its efficacy to modulate irregular flowering under Florida climate conditions in the presence of HLB, whether exogenous GA₃ further shortens the infection window of flower-targeting pathogens and enhances disease prevention awaits determination.

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Supplemental Table 1. Gene-specific primer sequences for gene expression analysis in ‘Valencia’ sweet orange with quantitative real-time polymerase chain reaction.

Gene name (gene symbol)	Reference	GenBank accession no.	Forward and reverse primer sequences (5' to 3')
<i>flowering locus t (FT)</i>	Goldberg-Moeller et al., 2013	XM_006480635.3	TGCATTGGTTGGTGACTGAT CGGAGGTCCCAGATTGTAAA
<i>leafy (LFY)</i>	Goldberg-Moeller et al., 2013	XM_006485103.2	AATGCCCAACCAAGGTGACTA AAAGCCCTCCTCAGTGCATTC
<i>apetalal (API)</i>	Goldberg-Moeller et al., 2013	XM_025100327.1	CCGCTCTCAAACACATCAGA TTAAGCAAGGAGGTGGCTGT
<i>suppressor of overexpression of constans1 (SOC1)</i>	Sousa et al., 2019	XM_006490906.3	AACAGCCAACGGAACAAAAC GCTCTTCTCCAGCTGACGTT
<i>actin7 (ACT7)</i>	Mafra et al., 2012, 2013	XM_006464503.2	GTTGGTTGACATGGAGAAG GACGGTTGAGTACAGAAATAAG
<i>dim1 homolog/yls8 (DIM1)</i>	Mafra et al., 2012, 2013	XM_006484463.2	CGAAACCTGTATGCAGATGG ACGGTTGAGGGATCGTAAAG

Supplemental Fig. 2. Phenology of 'Navel' sweet orange trees used in the current research based on field observations and the experimental design illustrating different gibberellic acid (GA) treatments over time: untreated control (GA-0) and monthly GA applications from September to January at the rate of 49 g·ha⁻¹ (GA-100).

Supplemental Fig. 3. Monthly average maximum and minimum air temperatures in Fort Meade and Haines City in 2017-18 and 2018-19.

Supplemental Fig. 4. Number of open flowers in untreated control (GA-0) 'Valencia' sweet orange trees and trees applied with gibberellic acid monthly from September to November, from September to December, and from September to January at the rate of 25 (GA-30, GA-40 and GA-50, respectively) and 49 g-ha⁻¹ (GA-60, GA-80 and GA-100, respectively) in Fort Meade (A) and Haines City (B) in 2017. Data are means $\pm$ SD of four biological replicates.

Supplemental Fig. 5. Number of elongated flower buds in untreated control (GA-0) 'Valencia' sweet orange trees and trees applied with gibberellic acid monthly from September to November, from September to December, and from September to January at the rate of 25 (GA-30, GA-40 and GA-50, respectively) and 49 g·ha⁻¹ (GA-60, GA-80 and GA-100, respectively) in Fort Meade in 2018 (A) and 2019 (B) and Haines City in 2018 (C) and 2019 (D). Data are means $\pm$ SD of four biological replicates. Within each treatment, asterisks indicate significant difference compared to the number on the first survey day based on Dunnett's test. Different lower and upper letters indicate significant differences ($P < 0.05$) among treatments on the individual survey days.