

Evaluation of Growth Regulator Inhibitors for Controlling Postbloom Fruit Drop (PFD) of Citrus Induced by the Fungus *Colletotrichum acutatum*

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Abstract. Postbloom fruit drop (PFD) of citrus is incited by the fungus *Colletotrichum acutatum* J. H. Simmonds and may result in young fruit drop and severe yield losses. Previous studies suggested that imbalance of growth regulators such as auxin, ethylene, and jasmonic acid (JA) plays an essential role in young fruit abscission. In this work, we determined the factors associated with fungal-induced fruit drop by testing compounds inhibitory to hormonal transport or biosynthesis. As assessed on sweet orange (*Citrus sinensis* Osbeck) and grapefruit (*C. paradisi* Macf.) for 4 years, we found that many auxin transport and action inhibitors such as 2,3,5-triiodobenzolic acid (TIBA), 2-(4-chlorophenoxy)-2-methyl-propionic acid (clofibrate), or quercetin and JA biosynthesis inhibitors such as salicylic acid (SA) and aspirin (methyl-SA) applied 7 d after *C. acutatum* infection resulted in higher percentages of young fruit retention compared with the water controls. The commercial products ReZist and Actigard, widely used as systemic acquired resistance (SAR) agents, also improved fruit retention. Furthermore, application of gibberellic acid (GA₃) on sweet orange, regardless of *C. acutatum* infection, significantly increased fruit retention. These commercial products may be very useful in managing this destructive disease of citrus in the field.

Postbloom fruit drop (PFD) of citrus is a severe problem for many citrus-producing areas in the humid tropics and subtropics of the Americas and has become a limiting factor for citrus production in some areas, including Belize, southern Mexico, Brazil, and Costa Rica (Timmer and Brown, 2000).

PFD first appeared in Florida in 1983 and caused significant yield losses in some years when environmental conditions were favorable for infection (McMillan and Timmer, 1989). PFD is incited by *Colletotrichum acutatum* J. H. Simmonds. The fungus infects flower petals, inciting necrotic lesions and petal fall within 4 to 5 d. The disease progresses so rapidly that control by fungicides is not always adequate despite the aid of tools such as a weather-based prediction model (Peres et al., 2004). Infection results in necrotic brown lesions on petals (blossom blight) and premature fruit drop (Timmer and Brown, 2000). The fungus infects almost all citrus species, particularly 'Navel', 'Natal', and 'Valencia' sweet oranges (*Citrus sinensis* Osbeck), and may also affect grapefruit (*C. paradisi* Macf.) and tangerines (*C. reticulata* Blanco) in the field (Timmer et al., 1994).

The most distinguishing characteristics of PFD are induction of young fruit abscission and formation of persistent enlarged calyces

(Timmer et al., 1994). The persistent calyces (commonly called 'buttons') attached on the peduncles are found only on the affected flower clusters and serve as diagnostic symptoms year round. In contrast, natural abscission of young fruit occurs in the junction between shoot and peduncle, often leaving no sign of the presence of flowers. Many fruit trees, including citrus, set large numbers of flowers and young fruit but undergo natural fruit drop to maintain favorable source/sink relationships as young fruit develop and mature (Bonghi et al., 2000). In citrus, more than 85% flowers are dropped naturally after bloom and only 0.5% to 2% flowers will eventually develop into mature fruit (Erickson, 1986). Early infection of citrus flowers by *C. acutatum* can result in complete yield loss. The process involved in young fruit abscission in a given species is complex and regulated by the levels of carbohydrates and hormonally driven signaling networks (Bangert, 2000; Bonghi et al., 2000; Goren, 1993; Sexton and Roberts, 1982; van Doorn and Stead, 1997). To gain more insight into understanding the interactions between *C. acutatum* and citrus, genetic systems for pathogen manipulation have been established (Chung et al., 2002) and used for identification and determination of genes required for fungal infection and symptom development (Chen et al., 2005). The types of growth regulators that might be involved in postbloom fruit drop were also investigated (Lahey et al., 2004). After infection by *C. acutatum*, we found that the content of ethylene and free IAA were significantly increased in citrus flowers. The levels of 12-oxo-phytodienoic acid (12-oxo-PDA, a precursor for JA biosynthesis) and jasmonic acid (JA) also drastically increased. In contrast, abscisic acid (ABA) levels did not change. Furthermore, Northern blot analyses revealed that genes encoding auxin-related proteins and 12-oxo-PDA reductase (JA biosynthesis), ACC oxidase, and ACC synthase (ethylene biosynthesis) were highly expressed in affected flowers after *C. acutatum* infection (Lahey et al., 2004; Li et al., 2003), suggesting that high amounts of auxin, ethylene, and JA may result from de novo biosynthesis in citrus flowers resulting from fungal colonization. Alternatively, high levels of growth regulators in the affected flowers might be, in part, produced by the fungal pathogen. Isolates of *C. acutatum* have been shown to produce various indole compounds in culture (Chung et al., 2003; Shilts et al., 2005). Thus, we hypothesized that imbalance of IAA, ethylene, and JA in the *C. acutatum*-infected flowers may account for young fruit drop. To test if growth regulators are involved in PFD, we conducted multiyear trials to evaluate compounds such as 2,3,5-triiodobenzolic acid (TIBA), 2-(4-chlorophenoxy)-2-methyl-propionic acid (clofibrate) or quercetin, JA biosynthesis inhibitors such as salicylic acid (SA), and gibberellic acid (GA₃) with documented ability in counteracting growth regulators to prevent young fruit abscission induced by *C. acutatum*.

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Materials and Methods

Biological materials. Grapefruit (*Citrus paradisi* Macf.) and Valencia sweet orange (*C. sinensis* Osbeck) trees in 10-gallon pots in a screenhouse located at the Citrus Research and Education Center, University of Florida (Lake Alfred, FL) were used for evaluations of the effects of hormone inhibitors for young fruit retention after fungal infection. The trees produced uniform inflorescences yearly in February and March and were irrigated, fertilized, and sprayed for pests to maintain trees in a healthy condition. *Colletotrichum acutatum* navel isolate (CaN) was recovered from infected flower petals of Navel orange in Florida (Chung et al., 2002). Fungal cultures were maintained routinely on potato dextrose agar (Difco, Detroit, MI).

Fungal inoculation. To prepare inoculum, fungal cultures were grown on potato dextrose agar under continuous fluorescent light for 5 to 7 days, and conidia were harvested by flooding with sterile water. After washing once with water, conidia were collected by centrifugation (5000 g for 5 minutes), and the concentration was determined with a Bright-Line hemocytometer (Hausser Scientific, Horsham, Pa.). Conidial concentration was adjusted to 5×10^4 per mL. The conidial suspension was sprayed onto grapefruit or sweet orange flowers at full bloom using a SpraTool sprayer (Crown, Woodstock, Ill.), and the branches with inoculated flower clusters were covered with a plastic bag for at least 12 h to maintain free moisture or high relative humidity and promote infection. The control branches were sprayed with water only and also bagged.

Screenhouse studies. To evaluate the efficacy of test compounds on young fruit retention, grapefruit and sweet orange trees producing uniform flowers between February and March were arbitrarily inoculated with conidial suspensions of *C. acutatum* CaN isolate prepared as described previously. A single application of benomyl (Benlate 50

WP) at $1.9 \text{ g}\cdot\text{L}^{-1}$ was made 1 day before inoculation as a fungicide standard treatment for comparison purpose. The total number of opened and unopened flowers was determined before inoculation. Test compounds were applied 7 dpi when most of young fruit are less than 8 mm in diameter, and fruit retention was evaluated ≥ 30 and >100 d after chemical application. Each treatment included at least 6 branches located in 5 different trees and the experiments were repeated on grapefruit in 2002 and 2003 and sweet orange in 2002 to 2005 using randomly chosen trees to overcome yearly variations in effects resulting from natural fruit drop of citrus. In 2005, over 500 flowers or young fruit on 10 branches of sweet orange were used to test the effects of SA ($10 \mu\text{M}$), ReZist (1 ml L^{-1}), and GA₃ (200 ppm) on young fruit retention. All opened and unopened flowers and young fruit were counted and combined before inoculation and after treatment by test compounds to minimize the variation resulting from natural fruit drop. Cumulative percentage of young fruit retention on selected branches was determined by dividing the total number of fruit retained after chemical application by the total number of fruit and flowers before inoculation.

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo.) unless otherwise indicated. N-(1-naphthyl)-phthalamic acid (NPA) was purchased from Chem Service (West Chester, Pa.). ReZist (2% copper, manganese and zinc, and other proprietary ingredients) was obtained from Stoller Chemical of Florida, Inc. (Eustis, Fla.). Actigard (benzothiadiazole) was obtained from Syngenta Crop Protection (Greensboro, N.C.). Gibberellin (GA₃) was purchased from SuperGrow (LaSalle, Québec, Canada). All compounds were dissolved in water to achieve the concentrations as indicated in the tables, and the pH of solutions was adjusted to 6.5 to 7.5 using 1N NaOH. All chemicals were applied to young fruit using a SpraTool sprayer until all materials were

completely covered. Surrounding tree branches were protected with cardboard to avoid spray drift during chemical application.

Data analysis. The significance of treatments was determined by analysis of variance using the PlotIT 2-way analysis (Scientific Programming Enterprises, Haslett, MI), and comparison of treatment means were compared using the Waller-Duncan *k* ratio *t* test ($P \leq 0.05$).

Results

2002 trials. The noninoculated controls (healthy) retained 8% and 7% of young fruit in sweet orange and grapefruit, respectively. Infected petals developed water-soaked, orange-brown lesions 4 to 5 d postinoculation (dpi). *C. acutatum* caused extensive sweet orange fruit drop (Table 1). Application of TIBA, clofibrate, SA, ReZist, or Actigard after *C. acutatum* infection increased fruit retention (Table 1). Similar chemical effects on young fruit retention were observed with grapefruit, except for ReZist, which did not prevent fruit drop. High dosages of TIBA (at concentration 1 mM), clofibrate ($>100 \mu\text{M}$), and SA (1 mM) caused twig dieback or defoliation of sweet orange and grapefruit.

2003 and 2004 trials. Fourteen different compounds at various concentrations were evaluated on sweet orange for prevention of young fruit drop incited by *C. acutatum* in 2003 and 2004 (Table 2). The noninoculated controls (healthy) retained 6.3% and 10.3% of young fruit in 2003 and 2004, respectively. *C. acutatum* infection resulted in less than 2% retention of young fruit. Benomyl sprayed 1 d before fungal inoculation reduced young fruit drop compared with the water control in 2003 trials. Like in 2002, application of TIBA, clofibrate, SA, ReZist, or Actigard on sweet orange resulted in a higher percentage of fruit retention as assessed 30 d after chemical application. Aspirin or quercetin also increased fruit retention in 2003 trials. Similarly, these chemicals prevented young fruit drop on sweet orange in 2004. Fruit retention was further determined 105 d after chemical application when fruit reached 4 to 5 cm in diameter, revealing that clofibrate, SA, ReZist, and Actigard, but not aspirin and quercetin, had long-term effects on fruit retention (Table 2). Compounds at higher concentrations did not improve fruit retention and tended to cause phytotoxicity such as yellowing, twig dieback, and defoliation. Other compounds such as 2,4-D, NPA, 2,3,5-TCPA, TCA, picloram, and HFCA provided some effects on young fruit retention but caused substantial phytotoxicity or inconsistent results in 2003 and 2004 and were not tested further.

2005 trials. In 2005, the effects of SA, ReZist, and GA₃ on young fruit retention were further evaluated (Table 3). As assessed 34 and 108 days after application, GA₃ significantly increased fruit retention in the absence of PFD. The branches inoculated

Table 1. Effect of test compounds on young fruit retention after inoculation with *Colletotrichum acutatum* isolate (CaN) on sweet orange and grapefruit in 2002.^z

Compounds ^y	Concn.	No. of fruit retained		Comments
		Sweet orange	(%) ^x (2002) Grapefruit	
None		8/96 (8.3) ab ^w	12/186 (6.5) a	Noninoculated
H ₂ O		0/87 (0) d	3/95 (3.2) c	Untreated
TIBA	1 μM	1/12 (8.3) ab	14/213 (6.6) a	
	100 μM	2/37 (5.4) c	7/127 (5.5) ab	Twig dieback
Clofibrate	1 μM	7/91 (7.7) bc	8/227 (3.5) c	
	1 mM	2/23 (8.7) bc	4/122 (3.3) c	Defoliation
SA	1 μM	6/47 (12.8) a	6/123 (4.9) bc	
	100 μM	2/33 (6.1) c	3/116 (2.6) c	Defoliation
ReZist	2.5 ml L ⁻¹	7/73 (9.6) ab	0/131 (0) d	
Actigard	0.1 g L ⁻¹	7/59 (11.9) a	11/209 (5.3) ab	

^zTest compounds were applied at the rates indicated 7 d postinoculation (dpi). The number of fruit retained was determined 30 d after treatment with test compounds.

^yTIBA = 2,3,5-triiodobenzolic acid. Clofibrate = 2-(4-chlorophenoxy)-2-methyl-propionic acid. SA = salicylic acid.

^xThe numbers of young fruit from 6 different branches before and after spraying with test compounds are to the right and left of the slash (/), respectively, and percent retention is shown in parentheses. The opened and unopened flowers were counted and combined before inoculation.

^wMeans followed by the same letter were not significantly different according to the Waller-Duncan *k*-ratio *t* test ($P \leq 0.05$).

Table 2. Effect of test compounds on young fruit retention after inoculation with *Colletotrichum acutatum* isolate (CaN) on sweet orange in 2003 and 2004.^z

Compounds ^y	Concn.	No. of fruit retained (%) [#]			Comments
		(2003) 30 d	(2004) 30 d	(2004) 105 d	
None		11/174 (6.3) bc ^w	12/116 (10.3) ab	2/116 (1.7) bc	Noninoculated
H ₂ O		1/79 (1.3) de	3/147 (2.0) e	0/147 (0) e	Untreated
TIBA	100 µM	20/157 (12.7) a	4/82 (4.9) c	0/82 (0) e	
Clofibrate	1 µM	11/127 (8.7) ab	2/40 (5.0) c	1/40 (2.5) bc	
	10 µM	9/131 (6.9) bc	6/61 (9.8) b	1/61 (1.6) bc	
SA	100 µM	20/219 (9.1) ab	7/159 (4.4) cd	3/159 (1.9) bc	Defoliation
	1 µM	5/90 (5.6) c	19/280 (6.8) bc	1/280 (0.4) de	
	10 µM	15/145 (10.3) ab	11/103 (10.7) b	8/103 (7.8) a	
	100 µM	5/110 (4.6) cd	7/195 (3.6) d	6/195 (3.1) b	Defoliation
ReZist	1 mM	10/133 (7.5) abc	ND		Defoliation
	10 mM	11/156 (7.0) bc	ND		Defoliation
	1 ml L ⁻¹	ND ^v	14/189 (7.4) bc	6/189 (3.2) b	
	2.5 ml L ⁻¹	2/127 (1.6) e	ND		
Actigard	5 ml L ⁻¹	ND	39/513 (7.6) bc	3/513 (0.6) d	
	10 ml L ⁻¹	9/97 (9.3) ab	51/516 (9.9) b	8/516 (1.6) bc	
	20 ml L ⁻¹	1/53 (1.9) e	ND		
Aspirin	0.1 g L ⁻¹	9/131 (6.9) bc	26/183 (14.2) a	4/183 (2.2) bc	
	1 g L ⁻¹	4/133 (3.0) d	10/295 (3.4) d	1/295 (0.3) de	
Quercetin	1 µM	ND	2/91 (2.2) e	0/91 (0) e	
	10 µM	ND	1/37 (2.7) e	0/37 (0) e	
	100 µM	9/129 (7.0) bc	1/229 (0.4) ef	0/229 (0) e	Defoliation
Picloram	1 µM	8/153 (5.2) c	5/80 (6.3) c	1/80 (1.3) c	
	10 µM	ND	13/216 (6.0) c	1/216 (0.5) d	
	100 µM	2/84 (2.4) de	6/45 (13.3) a	0/45 (0) e	Defoliation
2,3,5-TCPA	1 µM	4/97 (4.1) cd	2/64 (3.1) de	1/64 (1.6) bc	
	10 µM	ND	0/42 (0) f		
	100 µM	4/110 (3.6) cde	ND		Chlorosis
TCA	1 µM	12/156 (7.7) abc	ND		Defoliation
	100 µM	6/124 (4.8) cd	ND		Defoliation
2,4-D	1 µM	9/112 (8.0) abc	2/92 (2.2) e	0/92 (0) e	
	10 µM	ND	3/105 (2.9) de	0/105 (0) e	Defoliation
HFCA	1 µM	14/162 (8.6) ab	0/80 (0) f		Defoliation
	10 µM	ND	0/28 (0) f		Defoliation
NPA	1 µM	4/95 (4.2) cd	0/20 (0) f		Defoliation
	10 µM	ND	0/47 (0) f		Defoliation
Benomyl	1 µM	3/62 (4.8) cd	0/20 (0) f		Defoliation
	100 µM	2/76 (2.6) de	ND		Defoliation
	1.9 g L ⁻¹	9/146 (6.2) bc	ND		

^zTest compounds were applied at the rates indicated 7 d after inoculation. The number of fruit retained was determined 30 d or 105 d after treatment with test compounds.

^yTIBA = 2,3,5-triiodobenzonic acid. Clofibrate = 2-(4-chlorophenoxy)-2-methyl-propionic acid. SA = salicylic acid. Aspirin = methyl-SA. TCPA = 2,3,5-trichlorophenoxy acetic acid. 2,4-D = 2,4-dichlorophenoxyacetic acid. TCA = *trans*-cinnamic acid. HFCA = 9-hydroxyfluorene-9-carboxylic acid. NPA = N-(1-naphthyl)-phthalamic acid.

[#]The numbers of young fruit from 10 replicate branches before and after spraying with test compounds are to the right and left of the slash (/), respectively, and percent retention is shown in parentheses. The opened and unopened flowers were counted and combined before inoculation.

^wMeans followed by the same letter were not significantly different according to the Waller-Duncan *k*-ratio *t* test ($P \leq 0.05$).

^vNot determined.

with *C. acutatum* also retained significantly more fruit after treatment with GA₃, SA, or ReZist assessed at 34 d and 108 d after application.

Discussion

Previous studies using biochemical and molecular analyses have established strong links between *C. acutatum*-triggered fruit drop and phytohormones in citrus (Lahey et al., 2004; Li et al., 2003). Abscission of immature fruit driven by hormone imbalance may simply be a consequence of plant resistance responses. Studies were undertaken to further delineate the roles of growth regulators in fungal-induced fruit drop and to evaluate potential compounds that might be useful in controlling PFD.

Colletotrichum acutatum triggered severe young fruit drop. In the present study, we found that compounds such as TIBA (auxin transport inhibitor) (Okuda and Hirabayashi, 1998; Tsurumi and Ohwaki, 1978) and clofibrate (a putative auxin inhibitor) (Sosa-Morales et al., 1997; Wareing and Phillips, 1981) provided a higher percentage of fruit retention compared with the water controls on sweet orange and grapefruit in 2002, 2003, and 2004. The results provide evidence to support the hypothesis that auxin plays an important role in PFD. Natural flavonoids such as quercetin have been suggested to act as negative regulators of auxin transport (Brown et al., 2001). Application of quercetin or other auxin transport inhibitors (picloram, TCA, HFCA, NPA, TCPA, and 2,4-D) on sweet orange flowers 7 dpi also provided

some control over young fruit abscission in some years (Table 2). Low dosage of 2,4-D has been used for preventing preharvest fruit drop on sweet orange and grapefruit (Ali-Dinar et al., 1976). Unfortunately, many of these compounds caused severe defoliation or leaf yellowing particularly in young trees, greatly reducing their potential for commercial use.

Salicylic acid (SA) is an inhibitor of JA biosynthesis and function (Doares et al., 1995; Peña-Cortés et al., 1993). Application of SA on sweet orange and grapefruit controlled young fruit abscission in all years tested; however, higher concentration of SA (>1 mM) resulted in defoliation. Application of SA has also been shown to suppress 12-oxophytodienoate reductase gene expression that is involved in JA

Table 3. Effect of test compounds on young fruit retention after inoculation with or without *Colletotrichum acutatum* isolate (CaN) on sweet orange in 2005.^z

Treatment 1	Treatment 2 ^y	No. of fruit retained (%) ^x (2005)		Comments
		34 d	108 d	
H ₂ O	H ₂ O	56/618 (9.0) bc ^z	9/618 (1.5) b	Healthy
CaN	H ₂ O	33/719 (4.6) c	5/719 (0.7) c	Uncontrolled
H ₂ O	GA ₃	139/584 (23.8) a	12/584 (2.1) b	Noninoculated
CaN	GA ₃	67/337 (20.0) a	16/337 (4.8) a	
H ₂ O	SA	63/509 (12.4) b	7/509 (1.4) bc	Noninoculated
CaN	SA	63/445 (14.2) b	7/445 (1.6) bc	
H ₂ O	ReZist	72/493 (14.6) b	9/493 (1.8) b	Noninoculated
CaN	ReZist	38/451 (8.5) bc	14/451 (3.1) ab	

^zTest compounds were applied at the rates indicated 7 d after inoculation. The number of fruit retained was determined 34 d and 108 d after treatment with test compounds.

^yGA₃ = gibberellic acid (200 ppm); SA = salicylic acid (10 μM); ReZist (1 mL·L⁻¹).

^xThe total numbers of young fruit from 10 replicate branches before and after spraying with test compounds are to the right and left of the slash (/), respectively, and percent retention is shown in parentheses. The opened and unopened flowers were counted and combined before inoculation.

^zMeans followed by the same letter were not significantly different according to the Waller-Duncan *k*-ratio *t* test ($P \leq 0.05$).

biosynthesis (Lahey et al., 2004), indicating that SA is likely functioning as an inhibitor for JA biosynthesis. Application of methyl-SA (aspirin), however, had less effect on young fruit retention compared with SA in 2004 trials.

Two commercial products ReZist and Actigard that have been used to induce systemic acquired resistance (SAR) (Ryals et al., 1996) in a wide range of crops had strong effects on young fruit retention in the presence of *C. acutatum*. Higher concentrations of ReZist or Actigard, however, did not produce consistent results in fruit retention, likely as a result of the multiple modes of action. The exact function of these compounds contributed to fruit retention is uncertain. Preliminary studies suggested that application of ReZist or Actigard also suppressed transcript accumulation of auxin-responsive GH3-like protein and 12-oxophytodienoate reductase (data not shown). Thus, ReZist and Actigard may likely alter the levels of endogenous plant hormones and thereby reduce young fruit abscission.

To identify existing products that can be immediately used for managing PFD in the field, we also tested gibberellic acid (GA₃) for its effect of young fruit retention. Gibberellic acids are plant hormones that play various roles in plant growth and development (Ben-Cheikh et al., 1997; Richards et al., 2001). Early field studies have shown that external application of GA₃ prevented fruit drop on sweet orange and grapefruit (Ali-Dinar et al., 1976). In this study, a similar effect of GA₃ in promoting fruit retention was observed on sweet orange in the absence of *C. acutatum* (Table 3). Intriguingly, GA₃ exhibited a strong effect on fruit retention after *C. acutatum* infection and was superior to SA or ReZist. The function of GA₃ for young fruit retention remains uncertain, but likely opposes the action of other growth regulators (Harberd et al., 1998). Application of GA₃ has recently been shown to accelerate IAA metabolism in citrus fruit (Chamarro et al., 2001). The effect of GA₃ on young fruit

retention after *C. acutatum* infection warrants further investigation.

Collectively, research has shown that *C. acutatum*-induced postbloom fruit drop of citrus involves a complex signaling network controlled by diverse plant growth regulators (Lahey et al., 2004). In the present study, we have identified several compounds capable of retaining more young fruit after *C. acutatum* infection. These findings further suggest that *C. acutatum* disrupts the endogenous plant regulator balance of the host and leads to young fruit abscission. Although the effects of fruit retention were not consistent among the treatments and between years, likely as a result of the irregularity of flower settings, physiological variations (e.g., photosynthesis) in branches, and natural fruit drop, it is evident that the effect is genuine because many compounds such as TIBA, clofibrate, SA, and ReZist improved fruit retention in multiyear trails. In addition, the products identified from this study have wider application windows because they are targeted on fruit retention rather than on pathogen control per se and can be applied after symptoms develop, thereby providing broader alternatives for managing PFD. Further investigations to determine the levels of hormones after compound treatments will be imperative to elucidate the mechanisms leading to young fruit retention of citrus. Large-scale field trials in PFD management using GA₃, ReZist, or others will provide better evaluation for their effects on young fruit retention.

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