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Assessing the Potential for *Colletotrichum acutatum* as a Biological Thinning Agent for Florida Citrus

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Abstract

Colletotrichum acutatum, causal agent of postbloom fruit drop of citrus, and two induced *C. acutatum* mutants (3-3 and 3-2) were tested as potential agents for reducing fruit load on 'Valencia' sweet orange (*Citrus sinensis*) and 'Temple' tangor (*C. reticulata x C. sinensis*). Wild-type *C. acutatum* RST and a *C. gloesporioides* isolate were applied as conidial suspensions while induced mutants of *C. acutatum* (3-3 and 3-2), which produced few conidia in culture, and the wild-type isolate RST resulted in the characteristic blossom infection and persistent enlarged calyxes ("buttons") associated with postbloom fruit drop. The bloom period in 1999 was extremely dry and only the mycelial suspensions of RST resulted in significant formation of PFD buttons. In 2000, some rain occurred during bloom, and conidial suspensions of RST resulted in greater button formation than did the mycelial suspensions. No other treatments resulted in greater button formation than was observed in non-inoculated controls, and little natural PFD was observed. At harvest, there were no differences in fruit load or fruit size among trees inoculated with wild-type *C. acutatum*, mutant *C. acutatum* isolates, the *C. gloeosporioides* isolate and non-inoculated controls.

Reduction in cropload, known as thinning, is used to enhance profitability of several commercial tree fruits (e.g. apple (1), peach (*Prunus persica* (L.) Batsch) (7), and pear (*Pyrus communis* L.) (8). thinning has considerable potential value in citrus production since there is often an economic premium for larger fruit associated with moderate croploads (10), and some citrus cultivars become alternate bearing when they are permitted to set large crops (reviewed in 2). NAA is registered for thinning of citrus, but is quite expensive at the 250-500mg/L rates needed in Florida citrus (15). As in other fruits, use of NAA to thin citrus also has the disadvantage of inconsistent response, with virtually no thinning in some blocks despite high rates of NAA applied (9).

Postbloom fruit drop (PFD) of citrus caused by the fungus *C. acutatum* is a widely-distributed disease infecting flowers and inducing abscission of citrus fruitlets in Florida and the Caribbean, and many Central and South American countries (11). A typical symptom of PFD is persistent basal disks and calyxes, which resemble buttons and are commonly called PFD buttons (11). There is a significant negative relationship between the number of PFD buttons formed and the number of fruit produced in the same bloom period (13), and substantial crop losses sometimes result (11). This suggested that the PFD pathogen *C. acutatum* may have potential as an economical an practical biological agent for controlling citrus cropload. Such a biological agent would likely provide fewer regulatory difficulties and may be less sensitive to grove variables that the PGRs currently used for thinning. To be commercially acceptable, there must be minimal risk of excessive crop loss from severe PFD in subsequent seasons, and the number of buttons from the previous season is highly correlated with PFD severity the following year (11). In this study we assess the potential for use of

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C. Acutatum as a biological thinning agent by comparing a wild-type strain alongside induced auxotrophic mutants, which are likely to largely disappear from the grove prior to the next season's bloom.

Materials and Method

Isolates. The *C. acutatum* isolates used in this study were: 1) wild-type isolate RST obtained from PFD infected *Citrus sinensis* 'Navel' from Ft. Pierce, FL (6) and 2) *C. acutatum* mutants M 3-2 and M 3-3 which we induced by using the nitrous acid procedure (4). Mutants 3-2 and 3-3 are apparently auxotrophs for essentials nutrients nutrients since they grow slowly on potato-dextrose agar (PDA) and Snider and Raper's (5) complete standard medium (CSM), but do not grow on Snider and Raper's minimal medium, whereas the wild-type isolate of *C. acutatum* grows well on PDA, CSM and minimal medium. Wild-type *C. gloeosporioides* strain RSS, was isolated from infected citrus fruit (6) and was used for comparative purposes as a non-pathogenic related species.

Inoculum. Inoculum was prepared with spores or mycelia collected from cultures of the indicated fungi grown on or in CSM. Complete standard medium for agar plates contained: 20 g of Bacto-peptone; 20 g of glucose; 0.46 g of KH_2PO_4; 0.5 g of $MgSO_4$; and 20 g of Nobel agar. Complete standard medium for liquid cultures contained: 20 g of Bacto-peptone; 20 g of glucose; 0.4 g of KH_2PO_4; 0.5 g of $MgSO_4$. CSM-agar-plate cultures for sporulation were grown at 25-27°C for 2 weeks. CSM liquid cultures for producing mycelia were grown at room temperature for 15 to 18 days while being shaken at 100 rpm, and mycelia were collected on sterile cheesecloth to prepare for final suspension. Immediately prior to application, mycelial and spore suspensions were prepared with spore concentration at $1x10^6$ spores/ml (determined microscopically using a hemacytometer) and mycelia concentration at 50 g wet weight/liter in psyllium mucilloid diluent. the humectant kiluent was prepared by filtering an autoclaved solution of 5 grams of psyllium fiber per liter of deionized water and is an established method for enhancing infection when fungal pathogens requiring free water are used as biocontrol agents (3). For mutants of *C. acutatum*, only mycelial suspensions were used because they seldom produced spores. For the wild-type isolate of *C. acutatum*, RST, both spore suspensions and mycelium suspensions were used, while only spore suspensions were used for *C. gloeosporioides* (RSS).

Applications and experiment design.

Experiments were conducted on mature trees in the IRREC grove in Ft. Plerce, Florida, which were maintained on a minimal cultural program with no fungicide applications within one month of inoculations. Treatments compared were: non-treated controls; humectant control (in 1999 only); *C. gloeosporioides* (RSS) conidia; *C. acutatum* wild-type (RST) conidia; *C. acutatum* wild-type (RST) mycelia; and mycelia of two *C. acutatum* mutant strains (3-2 and 3-3). Trees of 'Valencia' sweet orange (*C. sinensis*) and 'Temple' (a tangor, *Citrus reticulata x C. sinensis)* at 40-60% bloom were sprayed with the undiluted inoculum suspensions using a hand-held CO_2 sprayer at 1 L/tree (in 1999 on Valencia) or at 4 L/tree of 4 x dilute inoculum using a commercial orchard sprayer (Rear's MFG. Co. Eugene, Oregon; in 2000 on 'Temple'). All applications were made in the early evening as dew was condensing to provide sustained initial exposure to free water and enhance probability of infection. In 1999, treatments were applied on April 5 with 12-14 hours of wet dew following applications but conditions were hot and dry on the subsequent two days, with a temperature range of 16-30°C. In 2000, treatments were applied on March 22 with 12-14 hours of wet dew following applications, and temperature ranged from 15-27°C on subsequent two days, with some rain on each day. Each treatment was applied to 6 trees in a randomized complete block design, with 1 tree per treatment per block, with tree size and vigor used as the blocking factor.

Survey of symptoms. Flower symptoms of PFD were surveyed in the two weeks fol-

lowing application. In 1999, only presence or absence of petal symptoms was observed. In 2000, 100 to 150 flowers were inspected randomly on each tree for evidence of infection. Persistent, enlarged calyxes were assessed in June of each year to permit easy discernment of these PFD buttons from calyxes remaining after normal fruit abscission. PFD buttons were counted within a 40x40 cm frame, using 5 randomly selected positions on the perimeter of each tree.

Cropping data. Fruit were harvested from all experimental trees at commercial maturity. All fruit were passed through a portable optical sizer (Autoline Inc., CA) which counted and determined diameter for all fruit, recording all data separately for each tree. Samples of fruit were collected and individual weights and diameters were used to establish a regression between diameter and weight. Number of fruit per tree, weight of fruit per tree, and mean fruit diameter were the parameters analyzed for each tree.

Statistical analysis. Data were analyzed using the GLM procedure of SAS (SAS Institute, Cary, N.C.); statistical significance is express at $p \leq 0.05$.

Results

PFD symptoms. Symptoms of PFD flower infection were evident on trees inoculated with RST conidia and mycelia, but not on trees inoculated with RST mutants, RSS, or on control (data not shown). In 1999 inoculation with the RST mycelial suspension increased formation of PFD buttons (persistent enlarged calyxes) but buttons produced from this treatment in 2000 were only significantly different from controls by contrast analysis (p=0.10) (Table 1). the spore suspension of RST (wild-type isolate of *C. acutatum*) significantly increased PFD button formation compared to controls in 2000 (Table 2), but not in 1999. Mutants, *C. gloeosporioides*, and humectant controls (1999 only) did not increase levels of PFD buttons beyond those observed in controls.

Cropping effects. No treatment significantly altered any aspect of cropping that was measured (Tables 1 and 2).

Discussion

Although PFD sometimes greatly decreases cropping in Florida (11), in most years weather conditions are not conducive to widespread PFD development. Timmer and Zitko (12) conducted a series of experiments to correlate environmental factors with severity of PFD. They concluded that inoculum availability was the primary factor responsible for disease variability, and that environmental conditions during bloom were usually suitable for disease development (temperature of 10-30°C and nightly leaf wetting of 10-14 hours) if inoculum was present. In addition, flowering is extended in Florida citrus and PFD doesn't appear to affect fruit that are already set (11). Therefore, the prospects appeared promising for using a weakened strain of *C. acutatum* as a biological thinning agent, by delivering high inoculum levels when fruit set is sufficient, but many susceptible flowers are still present.

Since *C. acutatum* spores germinate on citrus petals without forming appresoria (16) they should be functionally equivalent to sections of actively growing mycelium, making mycelial suspensions a reasonable means of delivering inoculum. It is interesting that the mycelial suspensions of the wild-type RST induced more PFD buttons that did the spore suspension in 1999 but fewer in 2000. In the hot and dry conditions of 1999, the mycelia may have produced infections more quickly than spores which would likely require additional time for germination and initial growth. In contrast, evidence of greater infection from RST spores in 2000 suggests that spores may be more effective in triggering infection when repeated rainfall conditions typically associated with PFD development.

Although conditions were not suitable for endemic PFD development in the two years of study, conditions were considered appropriate for infection to occur if inoculum were supplied, with temperatures ranging from 16-30°C and 12 to 14 hours of dew wetting following applications. While evidence of infection was only slight in 1999, trees treated with RST had fairly widespread floral symptoms of PFD in

Table 1. Response of 'Valencia' orange to treatment with mutants and wild-type isolates of *C. acutatum* (strain RST) and *C. gloeosporioides* (strain RSS) at bloom in 1999, Ft. Pierce, FL.

Treatment	Buttons per frame	Fruit per tree	Fruit diam. (mm)	Fruit yield per tree (kg)
Non-treated control	0.03 b	520 a	77 a	130 a
Humectant control	0.02 b	666 a	74 a	159 a
RSS conidia	0.05 b	670 a	72 a	149 a
RST mycelia	2.47 a	674 a	74 a	159 a
RST conidia	0.00 b	696 a	77 a	183 a
Mutant 3-2 mycelia	0.15 b	665 a	77 a	162 a
Mutant 3-3 mycelia	0.00 b	696 a	74 a	164 a

2000. It appears that the infection processes leading to fruit abscission and button formation may be more demanding than anticipated from the Timmer and Zitko (14) model, which was developed for natural infections and therefore was not intended to separate inoculum development from infection. These studies involved application of a known pathogen and uncharacterized mutants, making it necessary to conduct these trials on the research center. Trees used for these studies were previously used in other experiments which reduced yield uniformity. To compensate, trees were blocked by apparent similarity and treatments were applied in mid-bloom to potentially induce greater cropload reductions than would be desired commercially, but permit testing of the biothinning concept. In both years there was substantial variability in cropping between trees within and between treatments, so cropping means were statistically inseparable despite substantial numerical differences. However, since cropping means for control trees were always among the lowest observed, it is unlikely that a biological thinning effect was obscured.

The percentage of citrus flowers which set and carry fruit to harvest is quite small and research indicates that production declines by 6 fruit for every 100 PFD buttons (13). Therefore it is not surprising that the cropload was not affected even though PFD buttons were observed. It should be noted that *C. reticulata* and its hybrids (such as 'Temple') typically produce fewer PFD buttons than do oranges or grapefruit, and often display complete fruitlet abscission (11). This may explain the relatively small number of PFD buttons observed in 'Temple' despite the high incidence of flowers expressing symptoms.

The two years of trials described in this report provide little encouragement for further development of *C. acutatum* as a biological thinner, since even the wild-type strain did not significantly reduce dropping. However, better understanding of the PFD infection process or improved

Table 2. Response of 'Temple' orange (*C. reticulata x C. sinensis*) to treatment with mutants and wild-type isolates of *C. acutatum* (strain RST) and *C. gloeosporioides* (strain RSS) at bloom in 2000, Ft. Pierce, FL.

Treatment	Flowers with PFD symptoms (%)	Buttons per frame	Fruit per tree	Fruit diam. (mm)	Fruit yield per tree (kg)
Non-treated control	0 c	0.5 b	606 a	65 a	77 a
RSS conidia	0 c	1.0 b	574 a	64 a	74 a
RST mycelia	33 b	1.8 b*	785 a	66 a	111 a
RST conidia	44 a	6.1 a	863 a	64 a	105 a
Mutant 3-2 mycelia	0 c	0.7 b	730 a	64 a	90 a
Mutant 3-3 mycelia	0 c	1.4 b	516 a	66 a	70 a

*contrast analysis indicates that this value differs from the non-treated and RSS controls at p=0.10.

formulations for delivery may eventually permit use of this organism as an effective thinning agent.

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