Coronatine and Abscission in Citrus

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ABSTRACT. **Coronatine is a polyketide phytotoxin produced by several plant pathogenic *Pseudomonas* spp. The effect of coronatine on abscission in *Citrus sinensis* L. Osbeck 'Hamlin' and 'Valencia' orange fruit, leaves, fruitlets, and flowers was determined. Coronatine at 200 mg·L^{-1} significantly reduced fruit detachment force of mature fruit, and did not cause fruitlet or flower loss in 'Valencia'. Cumulative leaf loss was 18% with coronatine treatment. Coronafacic acid or coronamic acid, precursors to coronatine in *Pseudomonas syringae*, did not cause mature fruit abscission. Ethylene production in mature fruit and leaves was stimulated by coronatine treatment, and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) and 12-oxo-phytodienoate reductase (12-oxo-PDAR) gene expression was upregulated. A slight chlorosis developed in the canopy of whole trees sprayed with coronatine, and chlorophyll content was reduced relative to adjuvant-treated controls. Leaves formed after coronatine application were not chlorotic and had chlorophyll contents similar to controls. Comparison of coronatine to the abscission compounds methyl jasmonate, 5-chloro-3-methyl-4-nitro-pyrazole and ethephon indicated differences in ethylene production and ACO and 12-oxo-PDAR gene expression between treatments. Leaf loss, chlorophyll reduction and low coronatine yield during fermentation must be overcome for coronatine to be seriously considered as an abscission material for citrus.**

Coronatine is a polyketide phytotoxin produced by several plant pathogenic *Pseudomonas* spp. (Mitchell 1982; Völksch and Weingart 1998). In compatible host–pathogen interactions, coronatine was associated with virulence and symptom development. Coronatine induces a diffuse nonhost-specific chlorosis after application to many plants (Bender et al. 1999; Gnanamanickam et al., 1982; Mitchell and Young, 1978). Although the mode of action of coronatine is not known, recent work demonstrated that coronatine was immunolocalized to the chloroplast and induced proteinase inhibitor and chlorophyllase gene expression (Tsuchiya et al., 1999; Zhao et al., 2001). In addition to its phytotoxic effects, coronatine can also induce growth regulator-like effects such as hypertrophy and can stimulate ethylene production and tendril coiling (Ferguson and Mitchell, 1985; Gnanamanickam et al., 1982; Kenyon and Turner, 1992; Perner et al., 1994; Stelmach et al., 1999).

It has been reported that coronatine shares significant structural and functional similarities with jasmonic acid, its precursor 12-oxophytodienoate, and methyl jasmonate, which are plant growth substances important in octadecanoid signaling (Haider et al., 2000; Koch et al, 1999; Koda et al., 1996; Weiler et al., 1994). Components of the octadecanoid pathway have been shown to affect the regulation of defense responses (Penninckx et al., 1998; Rickauer et al., 1997), wounding (Benedetti et al., 1998), fruit ripening (Fan et al., 1998), and abscission (Miyamoto et al., 1997; Ueda et al., 1996). Our interest in octadecanoid compounds was in their potential as abscission-inducing agents for citrus. Both jasmonic acid and its methyl ester methyl jasmonate were shown to induce leaf abscission in bean explants without significant production of ethylene (Miyamoto et al., 1997; Ueda et al., 1996). In citrus, methyl jasmonate induced citrus mature fruit abscission when applied to the entire tree canopy (Hartmond et al., 2000a; Kender et al., 2001). However, unacceptable leaf abscission occurred in 'Valencia' orange, and this was associated with increased ethylene production in both leaves and fruit. Since coronatine and octadecanoid compounds can induce similar biological activities, we tested the ability of coronatine to cause abscission in citrus. Overall, our results indicate that coronatine induced mature fruit abscission, had low leaf loss, and did not cause immature fruit or flower loss.

Materials and Methods

BACTERIAL STRAINS AND GROWTH CONDITIONS. For all experiments described in this work we used *Pseudomonas syringae* pv. *glycinea* PG4180.N9 to produce coronatine. *Pseudomonas syringae* pv. *glycinea* PG4180.C0 and PG4180.B2 were used to produce the precursors to coronatine, coronafacic acid (CFA), and coronamic acid (CMA), respectively (Ullrich et al., 1994). Cells were routinely cultured on mannitol-glutamate-yeast extract medium (MGY; Keane et al., 1970) at 26 °C. For coronatine production, a previously described two-step fermentation protocol was used (Palmer and Bender, 1993). Briefly, for each fermentation batch, a loop of fresh culture was inoculated into 12 different tubes containing 10 mL MGY broth supplemented with 10 μg·mL^{-1} of kanamycin. Starter tubes were incubated in a rotary shaker at 26 °C and 220 rpm. After 48 h, cultures obtained a density of $\approx 1.0 \times 10^7$ cfu/mL (A_{600} 1.0). All starter cultures were combined and 25 mL were inoculated into 1-L baffled flasks containing 475 mL of Hoitink-Sinden medium (Palmer and Bender, 1993) supplemented with 10 μg·mL^{-1} kanamycin. Fermentation was carried out at 18 °C for 6 d at 220 rpm in a rotary shaker.

ISOLATION AND QUANTITATION OF CORONATINE. Coronatine was isolated from the fermentation media by partitioning against ethyl

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acetate and sodium carbonate as described by Palmer and Bender (1993). After drying *in vacuo*, coronatine was stored at –80 °C. Before application, the material was quantified by HPLC as described (Palmer and Bender, 1993).

Plant material. *Citrus sinensis* (L.) Osb. 'Hamlin' or 'Valencia' grown at the Citrus Research and Education Center, Lake Alfred, Fla., was used. Studies were conducted in the 1999, 2000, 2001, and 2002 seasons. Experiments with 'Hamlin' oranges were conducted in November, December, or January, whereas experiments with 'Valencia' oranges were conducted in March, April, May, or June. Average minimum and maximum temperatures during experiments with 'Hamlin' were 11.3 and 23.6 °C, respectively, and 18.6 and 34.1 °C, respectively, with 'Valencia'. No rain fell on trees within the first 3 d after application. Trees on 'Swingle' rootstock were 12 to 16 years of age and ranged from 3 to 5 m in height. In most experiments, coronatine or other abscission compounds were applied to isolated branches. Branch sprays consisted of five branches on each of three trees, with at least 10 fruit and 50 leaves per branch. Branch tests were replicated at least three times. Loss of 60-d-old fruitlets was evaluated in 'Valencia' orange using branch tests as described above. In one experiment conducted in 2002, coronatine was applied to entire trees. In this case, four trees of 'Valencia' orange were used. Three replicates of mature and young leaves (12 leaves/replication) were randomly removed from two trees at the north, south, east, and west canopy locations for chlorophyll analysis.

Abscission materials and application to citrus. Ethephon (Aventis, Ethrel, [2-chloroethyl] phosphonic acid), Ally (Dupont Chemical, metsulfuron-methyl, methyl 2-[(([(4-methoxy-6-methyl-1,3,5-triazin-2yl) amino] carbonyl) amino] sulfonyl) benzoate] and Kinetic adjuvant (Setre Chemical Co., Memphis, Tenn.) were purchased from Helena Chemical Company (Collierville, Tenn.). 5-Chloro-3-methyl-4-nitro-pyrazole (CMN-P; 17.18% active ingredient) was applied as formulated by Abbott Laboratories ≈30 years ago (Wilson et al., 1973). Methyl jasmonate (MJ) was purchased from Bedoukian Research Inc. (Danbury, Conn.). Coronatine, CFA or CMA were extracted from the fermentation media and quantified by HPLC as described by Palmer and Bender (1993).

Abscission materials were dispersed in 0.125% Kinetic immediately before application to the citrus canopy. Ethephon and CMN-P were applied at 200 mg active ingredient/L. Metsulfuron-methyl was applied at 2 mg active ingredient/L. Coronatine was applied at concentrations ranging from 0 to 300 $mg \cdot L^{-1}$; whereas CFA and CMA were applied at 100 $mg \cdot L^{-1}$. Treatments were applied to citrus until solutions began to run-off the canopy.

Measurements. Fruit detachment force (FDF) in kg was measured within 5 d after application of ethephon, coronatine, CFA, CMA or CMN-P and 10 d after application of metsulfuron-methyl using a Force Five pull-force gauge (Wagner Instruments, Greenwich, Conn.) as described (Hartmond et al., 2000b). Leaf loss in ethephon and coronatine treatments was measured 10 d after application of coronatine or ethephon. Leaf loss was expressed as a percentage by counting the total number of leaves at the beginning of each test and dividing by the number of leaves dropped. Fruitlet loss with coronatine, metsulfuron-methyl or CMN-P treatments was determined 22 d after application and expressed as percentage as described above. Chlorophyll was extracted from leaves of 'Valencia' and quantified according to the method outlined by Hipkins and Baker (1986). Analysis of variance and Duncan's multiple range test were performed using the SAS statistical package (SAS Inst. Inc., Cary, N.C.). Ethylene production from mature fruit and leaves of 'Hamlin' orange was measured by gas chromatography as described by Burns et al. (1999). Data were presented as means ± SE mean.

Gene expression analysis. Total RNA was extracted from fruit abscission zones, leaf abscission zones, and leaf blades of *Citrus sinensis* 'Hamlin' orange 24 h after application of coronatine, CMN-P, ethephon, 10 mM MJ, or adjuvant (Burns et al. 1998). Ten micrograms of total RNA were electrophoresed on a 1% formaldehyde-agarose gel and transferred to Hybond-N membranes (Amersham, Pharmacia, Biotech Inc., Piscataway, N.J.) by capillarity using 10× SSC as transfer buffer. Membranes were dried, cross-linked, and prehybridized in Perfect Hyb Plus hybridization buffer (Sigma Chemical Co., St. Louis, Mo.). RNA was hybridized with digoxigenin labeled cDNA probes (DIG Prime DNA Labeling and Detection Starter Kit II, Roche, Indianapolis, Ind.) following the manufacturer's instructions. Probes were generated using partial cDNAs sequences of ACO and 12-oxo PDAR genes previously isolated from citrus (Burns, 2002). Sequences were cloned into pGEMT-easy (Promega, Madison, Wis.) and used as DNA templates for PCR reactions. PCR amplification was performed with primers derived from the vector which flanked the insert DNA. PCR conditions were: initial denaturing at 94 °C for 5 min; 35 cycles denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, with a final extension step of 10 min at 72 °C. Hybridization signals were quantified using Quantity One Quantitation Software (Bio-Rad, Hercules, Calif.). Gene expression analysis was repeated twice with total RNA from each treatment.

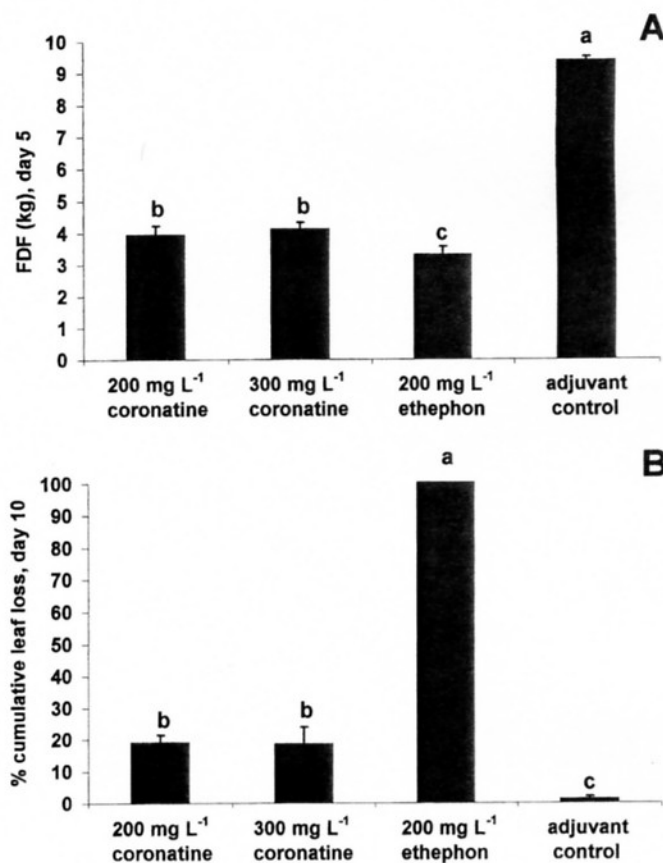

Fig. 1. The effect of coronatine on fruit detachment force (FDF) (**A**) and % cumulative leaf drop (**B**) in 'Hamlin' orange branch tests. Coronatine was applied at 200 and 300 $mg \cdot L^{-1}$, ethephon at 200 $mg \cdot L^{-1}$ and Kinetic adjuvant alone at 0.125%. Vertical bars represent SE mean. Mean separation by Duncan's multiple range test, $P < 0.05$.