Suppression of *Phytophthora* Infection in Citrus Infected with Viroids

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Abstract. Citrus viroid-induced resistance to *Phytophthora* infection in citrus was measured by the number of *Phytophthora* sporangia in ‘Rio Red’ grapefruit (*Citrus paradisi* Macf.) bait tissue infected with citrus viroids compared with non-inoculated controls. Different viroid isolates containing mixtures of viroids [*Citrus exocortis* viroid (CEVd), *Hop stunt* viroid (HSVd), *Citrus viroid III* (CVd-III), *Citrus viroid IV* (CVd-IV)] were designated by plant numbers and sources. Source 13E was associated with the lowest number of sporangia in bark, leaves, and roots used as baits, whereas CEVd E9, a known severe CEVd isolate, significantly reduced the number of sporangia in leaves and bark. Sources 1A, 2E, 3E, 4D, and 6E showed a significantly reduced number of sporangia on bark, leaves, and roots compared with healthy plants and 44A; however, their effect was not as pronounced as that of E9 and 13E. Sources 12E and 44A did not suppress sporangia production. Previous reverse transcriptase–polymerase chain reaction analysis showed that all source plants had mixed infections with several viroids, whereas 12E and 44A contained no viroids. In addition to confirming the earlier reports on the suppression of *Phytophthora* infection in general, our study showed significantly reduced *Phytophthora* sporangia development resulting from a number of viroids in mixed infection, but there did not appear to be any effect related to viroid species. To determine if concentration affected resistance to *Phytophthora*, phenolic acids were extracted. Extraction of phenolic acids with 80% ethanol was more efficient compared with 100% methanol and an acetonitrile–water mixture. High-performance liquid chromatography revealed no notable detection of salicylic acid in healthy and viroid-infected plants, but there was a small peak corresponding to salicylic acid in *Phytophthora*-infected and both viroid and *Phytophthora*-infected plants. Flavone was detected in all the source plants with a slight increase in *Phytophthora*-infected and both viroid and *Phytophthora*-infected plants. A peak corresponding to quercetin dehydrate was detected in *Phytophthora*-infected plants. Efficient use of the right viroid isolate(s) can result in suppression of *Phytophthora* infection of citrus.

Root rot caused by *Phytophthora nicotianae* and *P. citrophthora* is one of the most serious and economically significant diseases of citrus (Graham and Timmer, 1994; Timmer and Menge, 1988). *Phytophthora* spp. can infect almost all parts of the citrus plant causing root rot, gummosis, and foot rot of the trunk; damping-off of seedlings; leaf blight; and brown rot of fruit. The use of resistant rootstock is an effective way to control *Phytophthora* infection. Quantitative evaluation of root rot susceptibility has indicated that trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and

‘Swingle citrumelo’ (*P. trifoliata* × *C. paradisi* Macf.) are tolerant rootstocks (Widmer et al., 1988).

Viroids are infectious agents composed of covalently closed, single-stranded RNA molecules that undergo autonomous replication and have the ability to induce diseases in susceptible hosts (Diener and Raymer, 1971; Semancik, 1979). Citrus viroids are classified into: *Citrus exocortis* viroid (CEVd), *Citrus bent leaf* viroid (CBLVd), *Hop stunt* viroid (HSVd), *Citrus viroid III* (CVd-III), *Citrus viroid IV* (CVd-IV), and *Citrus viroid V* (CVd-V) (Duran-Vila et al., 1988). Citrus viroids cause two economically important diseases, exocortis caused by CEVd and cachexia caused by HSVd (Roistacher, 1991). Exocortis was first described in 1948 as a bark-shelling or scaling disorder in trees grown on trifoliate orange and some of its hybrids (Benton et al., 1949; Fawcett and Klotz, 1948). Later, this disease was demonstrated to be graft-transmissible (Benton et al.,

1950; Fawcett and Klotz, 1948). Cachexia was first described in 1948 (Childs, 1950) with specific disease symptoms of discoloration, gumming, and browning of phloem and bark cracking on ‘Orlando’ tangelo (*C. paradisi* Macf. × *C. reticulata* Blanco).

In Brazil, Rossetti et al. (1980) observed that exocortis-infected ‘Hamlin’ orange [*C. sinensis* (L.) Osb.] on Rangpur lime (*C. limonia* Osb.) rootstock resisted *Phytophthora* infection, but there were no consistent differences between the effects of mild and severe exocortis strains on *Phytophthora* lesions. Later, it was determined that the severe strains were exocortis and mild as were other viroids such as CVd-III.

Infection resulting from a pathogen induces changes in the host plant, including reinforcement of the cell wall by the formation of an impermeable deposit (Rossetti et al., 1980), accumulation of low-molecular-weight antifungal substances such as phytoalexins (Afeck and Szejnberg, 1988), and the appearance of new pathogenesis-related proteins (Afeck and Szejnberg, 1989; Antoniow et al., 1980). Some of the changes in the infection zone suggest that phenolic compounds may be the first line of defense (Matern and Kneusel, 1988) either as toxic products (free phenolic acids or their oxidation products, quinones) or as impedance in the invaded tissue (Bonhoff et al., 1987). An increase in phenols provides resistance for plants against fungal, bacterial, and viral infections (Matern and Kneusel, 1988). Soluble, low-molecular-weight phenolics are considered to have greater fungicidal activity compared with high-molecular-weight compounds (Harborne, 1989). Friend (1981) suggested that cell wall-bound phenolic acids alter the cell wall, creating a physical and chemical barrier against invading fungi. In citrus, phenolic compounds are produced in response to pathogen attack and stress factors such as ultraviolet radiation (Bonhoff et al., 1987; Broers and Jacobs, 1989; Manthey et al., 2000; Robbins, 1990). The production of antifungal phenolic compounds has been demonstrated in citrus fruits (Rodov et al., 1994), peels (Dubery et al., 1999), leaves (Harborne, 1989), and roots (Feldman and Hanks, 1968). Two antifungal compounds, xanthoxylol and scoparone, were found in the bark of lemon after infection with *P. citrophthora* (Afeck and Szejnberg, 1988, 1989, 1990, 1993, 1995; Afeck et al., 1986). Scoparone accumulation was found to be responsible for resistance against collar rot caused by *P. citrophthora*. However, scoparone levels did not show strong relation to root rot resistance in response to infection by *P. nicotianae* (Aucamp et al., 2000).

In this study, we investigated the possible suppression of *Phytophthora* in citrus resulting from the presence of citrus viroids. Through high-performance liquid chromatography (HPLC), we evaluated the associated changes in the level of phenols in healthy compared with viroid-infected, *Phytophthora*-infected, and both *Phytophthora* and viroid-infected plants and investigated the levels of sporangia formation in each category of these plants.

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Materials and Methods

Plant material for inoculation of viroids.

In this study, we used viroid source plants that were previously confirmed with the presence of the citrus viroids (Kunta et al., 2007b), including CEVd E9, an isolate that induces severe epinasty in Etrog citron (*C. medica* L.) (Baksh et al., 1984). Source plants 1A, 2E, 4D, and 13E contain CEVd, HSVd, and CVd-III, whereas 6E has all these viroids plus CVd-IV. Young leaves of source plants, 1A, 2E, 3E, 4D, 6E, 12E, 13E, non-inoculated control 44A, and healthy Rio Red grapefruit (*C. paradisi* Macf.) plants were collected, washed with water, dried, and weighed. The presence of viroids in these plants was previously confirmed by reverse transcription–polymerase chain reaction (RT-PCR) (Kunta et al., 2007b). Viroid extract was made by adding five times the weight of the leaves with deionized water and pulverizing the leaf tissue using a mortar and pestle. Young Rio Red grapefruit plants were inoculated with CEVd E9 viroid source by making slashes with a razor blade dipped in the viroid extract. The other sources were inoculated the same way. All the inoculation experiments were replicated three times. The inoculated plants were labeled and kept for 3 weeks in a greenhouse. Non-inoculated control plants were also kept under the same conditions. Seven of the inoculated plants were challenged with *P. nicotianae* by dipping the wounded plant roots in the liquid culture for 10 min. Additionally, the *P. nicotianae* inoculum was poured into the root zone of the potted plants. The challenged plants were then kept for 4 more weeks to initiate *Phytophthora* infection.

Extraction of phenolic acids using high-performance liquid chromatography. Phenolic acids were extracted from CEVd E9-infected, *Phytophthora*-infected, and both viroid and *Phytophthora*-infected grapefruit on sour orange (*Citrus aurantium* L.) rootstock along with healthy control plants. Roots of these plants were washed with water, dried, and pulverized using a mortar and pestle. To this powder, an acetonitrile–water (50:50) mixture was added, vortexed, filtered, and injected into HPLC. The same procedure was followed using 100% ethanol. Additionally, to extract phenolic acids using 80% ethanol, the roots were washed with water, oven-dried for 40 min at 90 °C, pulverized using a mortar and pestle, and stored at 4 °C until used. The powder (0.2 g) was treated with 80% ethanol for 6 to 7 h at 85 °C. The solution was vortexed for 1 min, sonicated for 5 min, and centrifuged for 5 min. The top layer of the solution was filtered twice using a cotton plug. Five milliliters of distilled water was added to this solution and subjected to rotoevaporation, and the residue was dissolved in 8 mL of 80% ethanol and injected into HPLC. The retention time for the peak was noted for and compared with a standard solution of known phenolic acid (Sigma-Aldrich, Milwaukee, WI). The solution corresponding to the salicylic acid, ferulic/gentisic acid, and

flavone was collected for further analysis with *Phytophthora*.

Biological analysis. *Phytophthora* growth was compared in leaves, bark, and roots of viroid-infected and healthy plants of Rio Red grapefruit on sour orange (*C. aurantium* L.). *Phytophthora nicotianae* (Kunta et al., 2007a) was isolated from soil samples through a standard leaf bait routinely used in our laboratory (Kunta et al., 2007a). One milliliter of soil suspension and leaf discs were also transferred to selective agar media plates with a final concentration of antibiotics 10 mg·L⁻¹ pimaricin, 200 mg·L⁻¹ vancomycin, 100 mg·L⁻¹ pentachloronitrobenzene, and 50 mg·L⁻¹ hymexazole. A gel slice with *Phytophthora* hyphae was transferred to V8 juice medium for further growth of the fungus and incubated in an incubator under dark at 15.4 °C for 2 d. The *Phytophthora* sporangia containing gel with the V8 juice medium was transferred to water and kept for 2 d. The zoospore release was induced by chilling the sporulating culture for 1 h and then keeping at room temperature for another hour. The zoospores were counted using a hemacytometer (Bright-Line; American Optical Corporation, Buffalo, NY). The leaves, bark, and roots of the viroid-infected and healthy plants were baited with 600 µL ($\approx 2 \times 10^5$ zoospores) of the zoospore solution of *Phytophthora*. Grapefruit leaf discs were used in leaf and bark assay and sour orange for root assay. After 4 d of incubation, the sporangia were counted under a microscope. The results of sporangial production in viroid-infected and healthy leaf discs were compared. The comparison of the effect of sporangial production between leaf, bark, and root tissue of viroid-infected and control grapefruit plants is represented in graphs using Sigma Plot.

Testing phenol effects on *Phytophthora* sporangia formation. The phenolic acids

collected by HPLC, flavone, salicylic acid, and gentisic acid, ferulic acid, were used to study the effects at different concentrations on sporangia formation. A 600-µL zoospore suspension was added to each 2 mL of the phenol solutions of series of 10-fold dilutions ranging from 1/10, 1/100, 1/1,000, 1/10,000, to 1/100,000. These were then used to bait Rio Red grapefruit leaf discs. After 4 d of inoculation, the number of sporangia was counted in each phenol solution at different concentrations.

Data analysis. In the first experiment, all the *Phytophthora* sporangia present in leaves, bark, and roots were counted. In the subsequent experiments, all the plant parts with more than 100 sporangia were scored as “100+.” In the data analysis, all “100+” scores were replaced by the average value of observations with more than 100 sporangia in the first experiment; the data were analyzed with the generalized linear model procedure (Proc GLM) of statistical analysis software (SAS Institute Inc., Cary, NC), and means were compared with the least significant difference method at $P = 0.05$. All sporangia counts were $\log(x + 1)$ transformed before analysis. The numbers of sporangia on leaves, barks, and roots between the viroids and the untreated control were compared by analysis of variance. The significant F-values were obtained; treatment means were separated using the Student Newman Keuls test.

Results and Discussion

In most of the healthy, viroid-infected, and *Phytophthora*-infected plants, a peak corresponding to ferulic acid was detected in HPLC analysis. There was a slight difference in peak area in both viroid and *Phytophthora*-infected plants. There was no notable detection of salicylic acid in healthy and

Fig. 1. Number of sporangia counted on leaves of grapefruit plants treated with different sources versus control (means followed by the same letter are not significantly different at $P = 0.05$, Student Newman Keuls test).

viroid-infected plants, but there was a small peak corresponding to salicylic acid in *Phytophthora*-infected and viroid- and *Phytophthora*-infected plants. Flavone was detected in all healthy, viroid-infected, *Phytophthora*-infected and both viroid- and *Phytophthora*-infected plants. However, there was a slight increase in the peak area of flavone in *Phytophthora* and both viroid- and *Phytophthora*-infected plants. A peak corresponding to quercetin dihydrate was detected in *Phytophthora*-infected plants.

Citrus viroid-induced tolerance to *Phytophthora* infection was measured by the number of *Phytophthora* sporangia in a group of seven Rio Red grapefruit plants on sour orange rootstock infected with citrus viroids and a non-inoculated control. Previous RT-PCR results showed that all the source plants have mixed infections with several viroids, whereas source plants 12E and 44A contained no viroids (Kunta et al., 2007b). There was a significant reduction in the number of *Phytophthora* sporangia in leaves, roots, and bark of viroid-infected plants compared with the healthy control. The number of *Phytophthora* sporangia varied with treatments on leaves ($F = 19.29$; $df = 9, 326$; $P < 0.0001$) (Fig. 1), roots ($F = 25.45$; $df = 8, 271$, $P < 0.0001$) (Fig. 2), and bark ($F = 18.23$; $df = 9, 312$; $P < 0.0001$) (Fig. 3). Plants infected with 13-E had the lowest number of sporangia in all three plant parts. The number of sporangia counted on leaves and bark of grapefruit infected with the CEVd isolate E9 was comparable to that of the source plant 13E. Treatment with source 44-A did not affect the number of sporangia compared with the untreated control on all three plant parts. Treatment with source 12E only significantly reduced the number of sporangia on roots and not on the bark and leaves. Although infection with the sources 1A, 3E, 4D, 2E, and 6E significantly reduced the number of sporangia relative to the control on leaves, bark, and roots, their effects were not as pronounced as those of the plant 13E and CEVd E9.

In all the experiments to detect differences in phenolic acids, flavone, salicylic acid, and gentisic acid/ferulic acid, it had been observed that at higher concentrations found, no sporangia were produced and at lower concentrations, the sporangia production increased drastically. However, there was not much difference in the number of sporangia produced with different phenolic acids. All inoculations were successful in producing infection based on RT-PCR analyses (Kunta et al., 2007b). The results obtained in this study and the statistical analyses clearly show that grapefruit plants infected with citrus viroids suppressed the formation of *Phytophthora* sporangia, especially with sources 13E and CEVd E9 plants. The results also suggest that there can be a possible relation between the concentration difference of phenolic acids in plants when they are infected with citrus viroids and *Phytophthora*. The results also show that there is a difference in the quantity of salicylic acid, flavone, and quercetin dihydrate in control, viroid-infected, *Phytophthora*-infected and

both viroid and *Phytophthora*-infected plants. Moreover, it is found that all the viroids may confer some degree of suppression against *Phytophthora*.

Rosetti et al. (1980) had observed that *Phytophthora* lesion size on Rangpur lime did not differ when plants were infected with a mild or severe exocortis strain(s). In our study, there was also no apparent effect related to viroid species. Although E9 (CEVd E9 only)-infected tissues had the lowest number of sporangia, the presence or absence of HSVd, CVd III, and CVd IV did not

significantly affect the number of sporangia. The only anomaly was in 12E-inoculated roots, in which a reduction of sporangia was found although inoculation was not successful (no viroid could be detected by RT-PCR). Citrus viroid-induced resistance of grapefruit plants toward *Phytophthora* may result in viroid-based control when the mechanism is fully understood.

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Fig. 2. Number of sporangia counted on roots of sour orange rootstock treated with different sources versus control (means followed by the same letter are not significantly different at $P = 0.05$, Student Newman Keuls test; *experiment not done).

Fig. 3. Number of sporangia counted on bark of grapefruit plants treated with different sources versus control (means followed by the same letter are not significantly different at $P = 0.05$, Student Newman Keuls test).

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