Effects of Fosetyl-Al and Phosphorous Acid on Scoparone, a Phytoalexin Associated with Resistance of Citrus to *Phytophthora citrophthora*

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ABSTRACT

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Two *Phytophthora*-resistant citrus species (macrophylla and sour orange) and two *Phytophthora*-susceptible citrus species (rough lemon and niva) were treated with fosetyl-Al or phosphorus acid and compared for production of scoparone and symptoms of infection of *Phytophthora citrophthora*. In macrophylla, sour orange, and rough lemon, concentrations of scoparone were two- to fourfold greater in inoculated branches treated with 300 μg of fosetyl-Al or 125 μg of phosphorous acid (H_3PO_3) per milliliter than in inoculated, untreated branches. Lesion length in these three species decreased more rapidly than in niva after treatments with 0–300 μg of fosetyl-Al or 0–125 μg of H_3PO_3 per milliliter, whereas lesion length in niva decreased sharply only with treatments of more than 500 μg of fosetyl-Al or 200 μg of H_3PO_3 per milliliter. Treatment with fosetyl-Al and H_3PO_3 did not influence scoparone concentrations in niva bark. ED_{50} values of fosetyl-Al and H_3PO_3 for mycelial growth of *P. citrophthora* were 55 and 7 μg/ml, respectively.

Root and collar rot caused by *Phytophthora* spp. is one of the most serious soilborne diseases of citrus (8). Application of protective fungicides has not provided adequate control of the disease. During the last 9 yr, significant progress in control of different diseases caused by *Phytophthora* spp. has been obtained with fosetyl-Al and metalaxyl (10,11,19). Previous studies have shown that fosetyl-Al (trade name Aliette, Rhone-Poulenc Sanitaire, Lyon, France) has little direct activity against mycelial growth of Oomycetes in vitro, although it has controlled diseases of citrus caused by these pathogens in the field and the greenhouse (7,10,11,16,21–23). These observations have led to the suggestion that fosetyl-Al is degraded to phosphorous acid (H_3PO_3) in the plant (9,12,13). Alternatively, fosetyl-Al may act indirectly by activating host defense mechanisms against pathogens (6,16–18).

Scoparone (6,7-dimethoxycoumarin), a phytoalexin associated with resistance of citrus to *P. citrophthora*, accumulates in citrus barks after inoculation with the pathogen. The ED_{50} for mycelial growth of *P. citrophthora* was 97 μg/ml in vitro (2–4). In some cases, scoparone accumulated to higher levels in infected bark treated with fosetyl-Al or H_3PO_3 than in infected bark that was not treated (1).

The purpose of this research was to determine the mode of action of fosetyl-Al in citrus. We report here evidence that low concentrations of fosetyl-Al act against *P. citrophthora* by increasing defense mechanisms against the pathogen and that higher concentrations are fungistatic.

MATERIALS AND METHODS

Plant and fungal material. The following 3-yr-old citrus seedlings from Kibutz Netzer Syreni Nursery, Israel, were grown outdoors and during the experiment were moved to the greenhouse and kept at 22–26 C: *Citrus jambhiri* Lush. (rough lemon), *C. reticulata* Blanco × *C. sinensis* (L.) Osbeck (niva), *C. macrophylla* Webster (macrophylla), and *C. aurantium* L. (sour orange). Rough lemon and niva are susceptible to *Phytophthora* and macrophylla and sour orange are resistant (2). The fungus *Phytophthora citrophthora* (Smith & Smith) Leonian (isolate C-16) was isolated from Lerrer Groves, Rehovot, Israel, in January 1983. The fungus was cultured on potato-dextrose agar (PDA) at 25 C to serve as inoculum.

Inoculation procedures. Five incisions, 3 mm long and 0.2–0.5 mm deep, were cut with a sterile scalpel in the bark of 3-mo-old citrus branches that were 25–30 cm long and 7–10 mm thick. A 3-mm-diameter disk cut from an actively growing culture of *P. citrophthora* was placed over the incision, fungal side downward, and the inoculated branch sections were incubated in a dark mist chamber at 20 C. Concentrations of scoparone in the bark and lesion lengths from the edge of the incisions to the end of the lesion were measured 4 days after inoculation. To test the effects of fungicides, 3-mo-old resistant or susceptible

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citrus branches were immersed in aqueous solutions containing fosetyl-Al (0–800 μg/ml) or H_3PO_3 (0–400 μg/ml) for 3 hr. The branches were then washed with tap water and inoculated as above. Advance of the pathogen (lesion length) and concentration of scoparone were measured 96 hr later (2).

Bioassay. The in vitro effects of fosetyl-Al and H_3PO_3 on mycelial growth of *P. citrophthora* were determined as follows: Increasing concentrations of fosetyl-Al or H_3PO_3 were added to cooled molten PDA immediately before it was poured into 9-cm-diameter plastic Petri plates. A 3-mm-diameter disk of *P. citrophthora*, taken from an actively growing colony on PDA, was placed fungal side downward in the center of each plate. Plates were incubated in darkness at 25 C for 8 days.

ED_{50} values were calculated from linear regression lines obtained by plotting the percent inhibition of mycelial growth area against the log concentrations of fosetyl-Al and H_3PO_3. The standard error of ED_{50} values was calculated from a linear regression analysis of fosetyl-Al and H_3PO_3 concentrations.

Experiments were repeated three times, and each treatment included five replicates.

RESULTS

Accumulation of scoparone and the advance of *P. citrophthora* in the bark of 3-mo-old citrus branches were measured after treatment with fosetyl-Al or H_3PO_3 and inoculation with *P. citrophthora*. In all citrus species except for niva, concentrations of scoparone in inoculated bark treated with fosetyl-Al (at 300 μg/ml) or H_3PO_3 (at 125 μg/ml) were two- to fourfold higher than in controls (inoculated and nontreated branches). Higher concentrations of fosetyl-Al or H_3PO_3 decreased scoporone concentrations (Figs. 1 and 2). Lesion length in macrophylla, sour orange, and rough lemon decreased more rapidly than in niva after treatments with 0–300 μg of fosetyl-Al or 0–125 μg of H_3PO_3 per milliliter (Figs. 1 and 2).

In niva, treatments with fosetyl-Al or H_3PO_3 did not affect scoparone concentrations, and the lesion length sharply decreased only at treatment levels above 500 μg of fosetyl-Al or 200 μg of H_3PO_3 per milliliter (Figs. 1 and 2). Treatment with fosetyl-Al and H_3PO_3 did not induce scoparone production in healthy bark in any of the species tested, and scoparone concentrations were 12–17.5 μg/ml fr. wt. as in controls (healthy and untreated branches). The regression analysis showed that inhibition of mycelial growth area of *P. citrophthora* was significantly correlated with increasing concentrations of fosetyl-Al ($r^2 = 0.960$, $P < 0.01$) and of H_3PO_3 ($r^2 = 0.949$, $P < 0.01$). The ED_{50} values of fosetyl-Al and H_3PO_3 for mycelial growth were 55 and 7 μg/ml, respectively (Fig. 3).

DISCUSSION

Various studies have reported that fosetyl-Al and H_3PO_3 induce resistance in plants against pathogens. Fosetyl-Al, which has some direct activity against Oomycetes in vitro, controls disease caused by these pathogens in the field and in the greenhouse. These studies assume, therefore, that fosetyl-Al acts by inducing host defense mechanisms against pathogens (5,6,16–18). Results of the present study suggest that fosetyl-Al and H_3PO_3 induce resistance in citrus by increasing the concentrations of scoparone in macrophylla, sour orange, and rough lemon (Figs. 1 and 2). Other studies indicate that fosetyl-Al has no effect on resistance (9,12,13). These researchers assume that fosetyl-Al penetrates the plant and is degraded to H_3PO_3, which is very toxic to *Phytophthora* spp. and inhibits the pathogen as a fungistat. Results of the present study further suggest that when fosetyl-Al penetrates the tissue, it is degraded to H_3PO_3. Scoparone accumulation and decrease of lesion length are similar after treatments with either fosetyl-Al or H_3PO_3 (Figs. 1 and 2), but the concentration of H_3PO_3 needed for the same effect as that of fosetyl-Al is 41%. This is a good indication that fosetyl-Al is degraded to about 41% H_3PO_3 in vivo. Results of this study show as well that H_3PO_3 is much more toxic to *P. citrophthora* than fosetyl-Al in vitro (Fig. 3).

Saindrenan and Bompeix (20) reported that the concentration of H_3PO_3 that was found at the front of mycelial progression

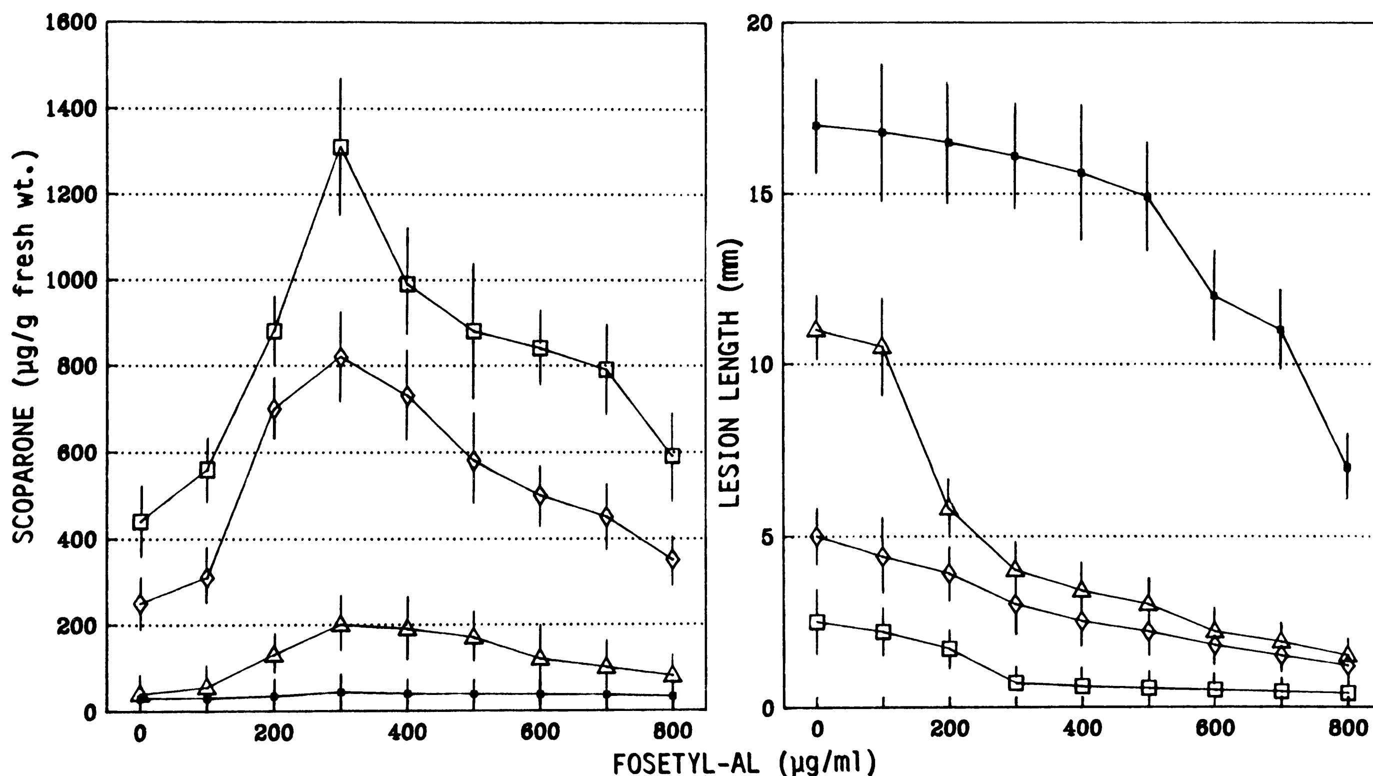

Fig. 1. Accumulation of scoparone (left) and lesion length (right) in barks of the resistant citrus species macrophylla (□) and sour orange (◇) and the susceptible species rough lemon (△) and niva (■) 96 hr after inoculation with *Phytophthora citrophthora*. Branches were treated with the fosetyl-Al 3 hr before inoculation. Vertical bars are standard errors.

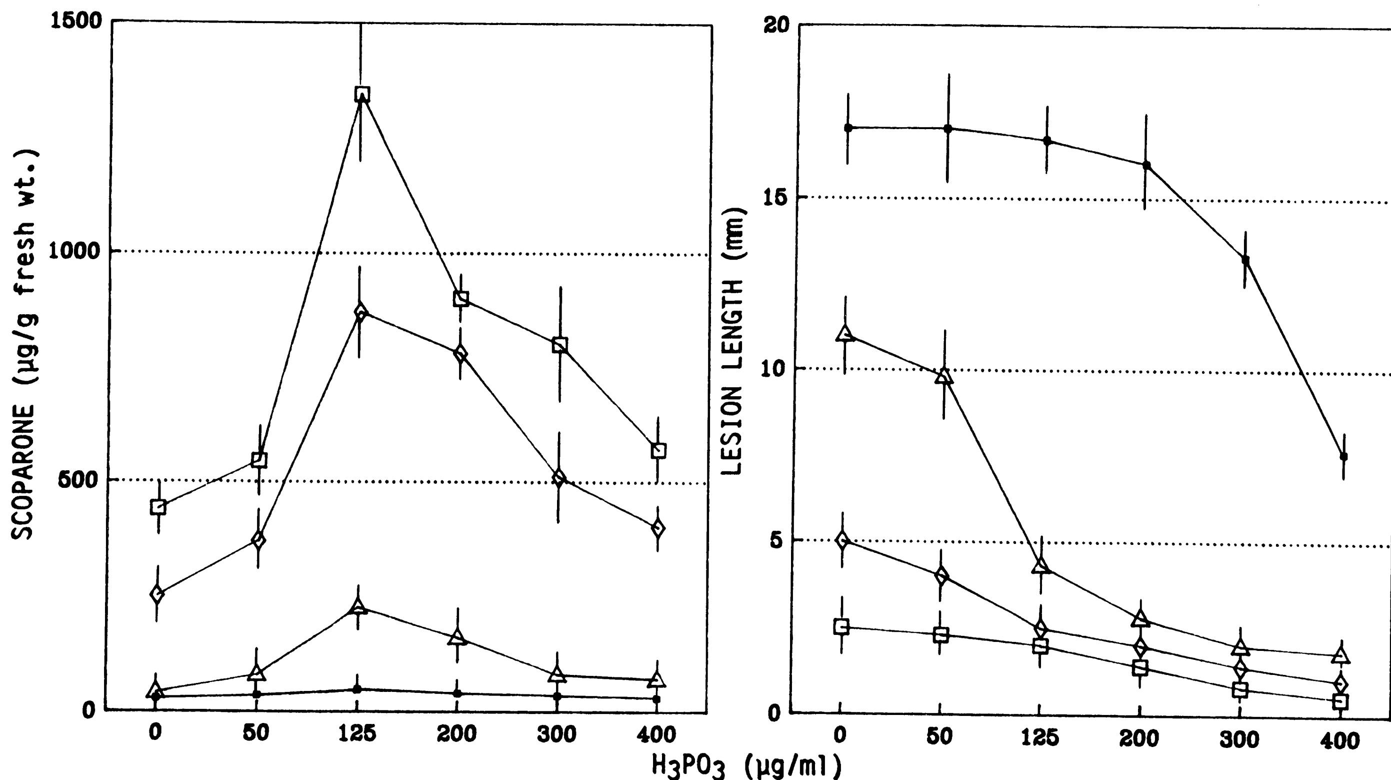

Fig. 2. Accumulation of scoparone (left) and lesion length (right) in citrus bark of the resistant species macrophylla (□) and sour orange (◇) and the susceptible species rough-lemon (△) and niva (■) 96 hr after inoculation with *Phytophthora citrophthora*. Branches were treated with H_3PO_3 3 hr before inoculation. Vertical bars are standard errors.

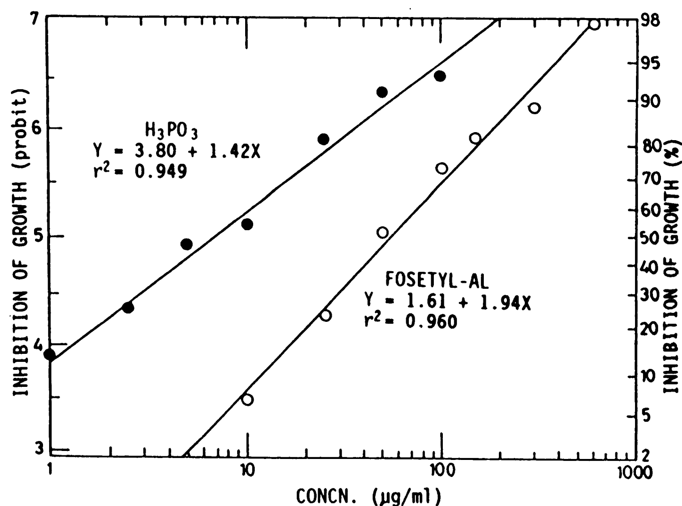

Fig. 3. Dosage-response of *Phytophthora citrophthora* mycelial growth to log concentration of fosetyl-Al (○) and H_3PO_3 (●), expressed as a linear regression.

in *Vigna* leaves inoculated with *P. cryptogea* and treated with fosetyl-Al was only one tenth of the in vitro ED_{90} value. Therefore, H_3PO_3 cannot be responsible for stopping the parasitic growth in vivo. On the other hand, Fenn and Coffey (12,13) reported that the high activity in vitro of H_3PO_3 and fosetyl-Al specifically against *Phytophthora* closely parallels their in vivo behavior and provides good circumstantial evidence of a direct mode of action against the pathogen. In contrast, Guest (14–16) reported that fosetyl-Al displays a little direct activity against mycelial growth of Oomycetes and that no evidence was found to indicate that it is converted to a more toxic derivative in the plant. This does not exclude the possibility that fosetyl-Al has more subtle effects on growth of the pathogen in vivo, but the weight of evidence suggests that the hypersensitive-like response results from an effect of fosetyl-Al on the host and that this does play a role in limiting the spread of the pathogen in treated plants.

This research has led to the suggestion that fosetyl-Al affects *P. citrophthora* in vivo in two ways: at low level treatments it increases the host defense mechanisms, and at higher levels it acts directly as a fungistat. In the citrus species macrophylla, sour orange, and rough lemon, maximum production of scoparone is stimulated by 300 µg of fosetyl-Al or 125 µg of H_3PO_3 per milliliter, but the concentration of scoparone in niva remains low (Figs. 1 and 2). This is explained by the "potential of resistance" (the particular selection of plant to produce scoparone) that these three species have and niva has not. Scoparone, which is associated with resistance (2,4), and the fungistat both have an effect on *P. citrophthora* in the citrus plants with the potential of resistance, whereas in niva only the fungistat has an effect directly on the fungus. Thus, the concentrations of fosetyl-Al and H_3PO_3 that are needed to stop the advance of the pathogen in macrophylla, sour orange, and rough lemon are lower than in niva (Figs. 1 and 2). To support this hypothesis, we assume that in vitro and in vivo ED_{50} values are similar and that lesion length in vivo reasonably reflects fungal growth in vitro. From Figures 1 and 2 it appears that 50% lesion length in niva seedlings on which fosetyl-Al and H_3PO_3 directly affect the pathogen, occur at an application concentration of about 750 µg of fosetyl-Al and at 350 µg of H_3PO_3 per milliliter. In vitro ED_{50} values for fosetyl-Al and H_3PO_3 are 55 and 7 µg/ml, respectively (Fig. 3). This suggests that only 55 µg of fosetyl-Al and 7 µg of H_3PO_3 per milliliter are present when ED_{50} is reached in vivo. If one assumes from this that only about 2% of the applied H_3PO_3 or 7.3% of the applied fosetyl-Al enters the tissue, then the peak scoparone production is estimated to occur at about 22 µg of fosetyl-Al per milliliter and 2.5 µg of H_3PO_3 per milliliter, which is equivalent to only ED_{20} and ED_{25} values, respectively, in vitro (Fig. 3).

Treatments of inoculated macrophylla, sour orange, and rough lemon with fosetyl-Al at 800 µg/ml or H_3PO_3 at 400 µg/ml did not increase the concentration of scoparone in vivo. The effect of these concentrations on scoparone production is similar to

that in control treatments with no fosetyl-Al and H_3PO_3 (Figs. 1 and 2). This is explained by the fact that treatments with 800 μg of fosetyl-Al or 400 μg of H_3PO_3 per milliliter inhibit the physiological activities of *P. citrophthora* (Fig. 3). These two compounds alone, e.g., treatment of healthy branches, cannot stimulate scoparone production.

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