Further Evidence for the Direct Mode of Action of Fosetyl-Al and Phosphorous Acid

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ABSTRACT

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Detached tomato leaflets were inoculated with *Phytophthora capsici* and floated on either water, fosetyl-Al, phosphorous acid (H_3PO_3), or metalaxyl, or one of these in combination with aminooxyacetic acid (AOA) or glyphosate. Glyphosate did not affect lesion size in any of the treatments. Compared to the water control, AOA caused a 28% increase in lesion length in leaflets inoculated with *P. capsici*. Fosetyl-Al (260 μg/ml), H_3PO_3 (180 μg/ml), and metalaxyl (5 μg/ml) inhibited lesion expansion, either in the presence or absence of 0.08 mM AOA. However, in the presence of AOA, lower concentrations of both fosetyl-Al (130 μg/ml) and H_3PO_3 (90 μg/ml) were less effective in reducing lesion size. With *P. capsici* in liquid culture, 0.08 mM AOA caused a 62% reduction in uptake of H_3PO_3 over 3 hr. A laboratory-developed mutant (H36) of *P. capsici* which was H_3PO_3-tolerant in vitro was also insensitive to both H_3PO_3 and fosetyl-Al in vivo.

Additional key words: aluminum tris-*O*-ethyl phosphonate.

The mode of action of the systemic fungicide fosetyl-Al (aluminum tris-*O*-ethyl phosphonate) is as yet unknown. In initial studies with fosetyl-Al researchers were only able to detect low in vitro antifungal activity against sensitive fungi (3,8,11,19,22–24, 26,28–30) and therefore oriented their studies towards the possibility that this compound acted indirectly against the target organism by stimulating host defense mechanisms (4–6,10,13–16,18,20, 22,27,29–31). Several papers have been published in which the results are interpreted as evidence that fosetyl-Al has an indirect mode of action which involves stimulation of host resistance mechanisms (4–6,14–16,20,29). Recently however, it was reported that ethyl phosphonates such as fosetyl-Al, and particularly a breakdown product (6,27,29) phosphorous acid (H_3PO_3), are inhibitory at low concentrations to mycelial growth of some species of *Phytophthora*, especially when grown on a medium containing low phosphate (9,12). In addition, several H_3PO_3-tolerant mutants of *P. capsici* have been produced in vitro by chemical mutagenesis and these were also tolerant to both H_3PO_3 and fosetyl-Al in vivo (7). Thus, there is now increasing evidence that the mode of action of fosetyl-Al and H_3PO_3 may involve a direct effect on the pathogen.

In a study of the host-parasite interaction between *P. capsici* and tomato it was reported that aminooxyacetic acid (AOA) and glyphosate, presumptive inhibitors of phenylpropanoid synthesis (1,2,17,25), reversed the inhibition of disease development by fosetyl-Al (5,13). This probably represents the strongest evidence presented to date that fosetyl-Al can act by stimulating host defense mechanisms.

The purpose of the research reported in this paper was to reexamine the tomato-*P. capsici* host-parasite system using both H_3PO_3 and fosetyl-Al as antifungal compounds.

MATERIALS AND METHODS

Materials used. Tritium-labeled H_3PO_3 (sp. act. 200 mCi/mmol) was custom-synthesized by Amersham, Arlington Heights, IL. It was synthesized by the reaction of phosphorous trichloride with tritiated water. The tritium label was bound to the phosphorus atom of H_3PO_3. A preliminary NMR study with H_3PO_3 and D_2O indicated that exchange of the phosphorus-bound tritium would not be a problem. Glyphosate [*N*-(phosphonomethyl)glycine] as the free acid was obtained from Monsanto, St. Louis, MO. Nonradioactive H_3PO_3 was obtained in crystalline form from Fisher Scientific, Pittsburg, PA, and aminooxyacetic acid (AOA) from Sigma, St. Louis, MO. Solutions in the various treatments were adjusted to a pH of 6.5 with 0.1, 1.0, or 10.0 N KOH prior to their use.

The wild-type (P1319-1) and H_3PO_3-tolerant (H36) isolates of *P. capsici* used in this study were single-spore cultures from a previous study by Bower and Coffey (7). In a previous study, the in vitro EC_{50} values were 37 μg/ml H_3PO_3 and 415 μg/ml H_3PO_3 for P1319-1 and H36, respectively (7). Tomato cultivar Casino Royale was used in all in vivo experiments. Tomato plants were grown in the greenhouse for 3 wk in 6-cm-diameter peat pots containing UC mix (12). Seedlings were then transplanted into 3.8-L metal containers containing UC mix. When tomato plants were 6–8 wk old, nonterminal leaflets, from the third to fifth expanded leaf below the plant apex, were selected for uniformity and detached from the main stem by cutting the petiole with a razor blade.

Inoculation and chemical treatment of tomato leaflets. Detached tomato leaflets were immediately sprayed with a fine mist of distilled water and inoculated at the leaf base, on the adaxial surface with an inverted agar plug (4 mm), taken from the margin of a 4-day-old colony of *P. capsici* growing on V8C agar (10% V8 juice, 1% $CaCO_3$, clarified by centrifugation) at 24 C in the dark. Inoculated leaflets were then floated adaxial side up on 15 ml of distilled water, fosetyl-Al, H_3PO_3, or metalaxyl, or one of these in combination with AOA or glyphosate in petri plates. Leaflets were then incubated in a growth chamber at 25 C under a daily 12-hr photoperiod. Light was from Sylvania Grolux lights (160 W) giving a photon flux density (PFD) of 170 $\mu E/m^2/sec$ at the surface of the petri plates. After 3 days of incubation, the lesion lengths were measured. Five leaflets were used per treatment and all experiments were repeated at least twice.

Uptake of H_3PO_3 by tomato leaflets and the resultant antifungal activity. The uptake of H_3PO_3 by detached tomato leaflets was determined under the above conditions, except that the leaflets were not inoculated with *P. capsici*. Leaflets were floated for 24 hr on H_3PO_3 solutions of varying concentrations (15 ml per petri

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plate) to which a constant amount of [^{3}H]-H_3PO_3 was added (0.038 μCi/ml of a 200 mCi/mmole solution). Following 24 hr, to allow uptake, 0.5 g fresh weight (leaflets saturated) of leaf tissue was placed in a screw-cap vial, frozen by adding liquid nitrogen, and ground to a powder with a glass rod. Distilled water (4.4 ml) was added and the vials were laid on their sides and agitated on a reciprocal shaker at 180 strokes (3.5 cm) per min for 1 hr. The contents of the vials were then centrifuged at high speed in an IEC clinical centrifuge for 10 min and 200 μl of the resulting supernatant was suspended in 3.7 ml of liquid scintillation fluid (Beckman Ready-Solv HP/b High Performance for aqueous samples) and counted in a Beckman model 7500 liquid scintillation counter. Counts were converted to μg of H_3PO_3 taken up per gram fresh weight of leaf.

To correlate the in vivo concentration of H_3PO_3, as determined by the radioisotopic method, with the level of inhibition of lesion development, a parallel experiment was performed simultaneously with the uptake experiment. In this parallel experiment, similar leaflets were also treated for 24 hr with nonradioactive H_3PO_3, inoculated with a 4-mm-diameter agar plug from a culture of *P. capsici*, and floated on water. Three days later, lesion lengths were measured. EC_{50} values for inhibition of lesion expansion were obtained from regression lines plotting lesion length versus log concentration H_3PO_3.

EC_{50} value in vitro for H_3PO_3. The in vitro EC_{50} value for P1319-1 was determined by adding H_3PO_3, adjusted to pH 6.5 with KOH, to cooled molten V8C agar, immediately prior to pouring into 8.5-cm-diameter plastic petri plates. A 4-mm-diameter agar disk, taken from an actively growing colony on V8C agar, was placed fungal side downward in the center of each plate. Plates were incubated in the dark at 24 C for 4 days. Radial growth was determined by measuring colony diameters at two points on each petri plate, computing the average value, and subtracting the diameter of the fungal plug. The EC_{50} value was obtained from a regression line by plotting colony diameter versus log concentration H_3PO_3.

Determination of phosphate levels in tomato leaves and V8C medium. Inorganic phosphate levels in tomato leaflets were determined by extracting phosphate according to the extraction procedure described above for the H_3PO_3 uptake experiments. The phosphate content of tomato leaflet extracts and of V8C broth was then determined with a Technicon autoanalyzer (Tarrytown, NY) based on a phosphorus-molybdenum blue complex and absorption at 660 nm.

Effects of AOA on uptake of H_3PO_3 by *P. capsici*. To determine H_3PO_3 uptake, *P. capsici* was cultured at 24 C in the dark for 4 days on V8C agar in 8.5-cm-diameter petri plates and then placed for 3–4 days at 24 C under fluorescent lights with a PFD of 17 $\mu E/m^2/sec$ at the culture surface. Ten milliliters of chilled (5 C), sterilized, distilled, and deionized water was added to each plate which was then placed at 5 C for 20 min and subsequently incubated at 24 C for 40 min to allow for zoospore release. The zoospore suspension was removed with a sterile syringe and collected in a beaker. Zoospore concentrations were estimated by agitating 3 ml of the zoospore suspension with a vortex to induce encystment. The number of cysts was then counted with a hemacytometer. Sufficient motile zoospores were then added to modified Ribeiro's liquid medium (21 (β-sitosterol was omitted and 0.084 mM KH_2PO_4 was added) to give a final concentration of 1.0 or 2.0 $\times 10^5$ zoospores per milliliter, depending on the experiment. After zoospores were added to the liquid medium, the resulting spore suspension was stirred vigorously for 1 hr with a magnetic stirring bar after which the suspension was placed on a reciprocal shaker at 102 (3.5 cm) strokes per minute. After 12 hr, 10-ml samples of the fungal culture suspension were removed with a widemouthed pipette for dry weight determination.

Uptake of H_3PO_3 by the fungal culture suspension was determined by adding unlabeled H_3PO_3 at 50 μg/ml (adjusted to pH 6.5 with KOH and sterilized by Millipore filtration [pore size = 0.2 μm]) and 0.038 μCi/ml of [^{3}H]-H_3PO_3, to the fungal suspension. In some treatments, AOA was added from a stock solution at this time. The culture suspensions were again placed on the reciprocal shaker and the amount of H_3PO_3 taken up at various times was determined by filtering 10-ml samples of the fungal suspension through Whatman GF/A filter disks (2.4-cm diameter) and rinsing them four times with 10-ml aliquots of 0.084 mM KH_2PO_4. The filter plus mycelium was then placed in a counting vial to which 3.7 ml of liquid scintillation fluid (Beckman Ready-Solv HP/b High Performance for aqueous samples) was added and counted in a Beckman model 7500 liquid scintillation counter. The experiment was repeated twice.

RESULTS

In vivo activity of H_3PO_3, fosetyl-Al, and metalaxyl in controlling H_3PO_3-sensitive and -tolerant isolates of *P. capsici*. The EC_{50} values for the sensitive isolate (P1319-1) for inhibition of lesion expansion in tomato leaflets were 51 μg/ml H_3PO_3 and 56 μg/ml fosetyl-Al (Table 1). A laboratory-derived H_3PO_3-tolerant isolate (H36) was relatively insensitive to H_3PO_3 and fosetyl-Al (EC_{50} values of >348 μg/ml and >500 μg/ml, respectively, for inhibition of lesion development) (Table 1). Lesion size caused by H36 was reduced by only 20 and 29% with H_3PO_3 and fosetyl-Al, respectively, at the highest concentrations tested (H_3PO_3 at 348 μg/ml and fosetyl-Al at 500 μg/ml). Both the sensitive and the

TABLE 1. Effective concentration (EC_{50}) values for inhibition of lesion expansion in tomato leaflets of the cultivar Casino Royale inoculated with a phosphorous acid (H_3PO_3)-sensitive (P1319-1) or an H_3PO_3-tolerant isolate (H36) of *Phytophthora capsici*

	EC_{50}[x] (μg/ml)		
Isolate	Fosetyl-Al	Metalaxyl[y]	H_3PO_3
P1319-1 (sensitive)	51	56	2.9
H36 (tolerant)	>348[z]	>500[z]	2.7

[x] EC_{50} values for inhibition of lesion expansion were obtained from regression line equations for a plot of percent inhibition versus log concentration.

[y] Metalaxyl data are from a separate experiment.

[z] Values shown are the highest concentrations used in the experiment; they resulted in less than 50% inhibition.

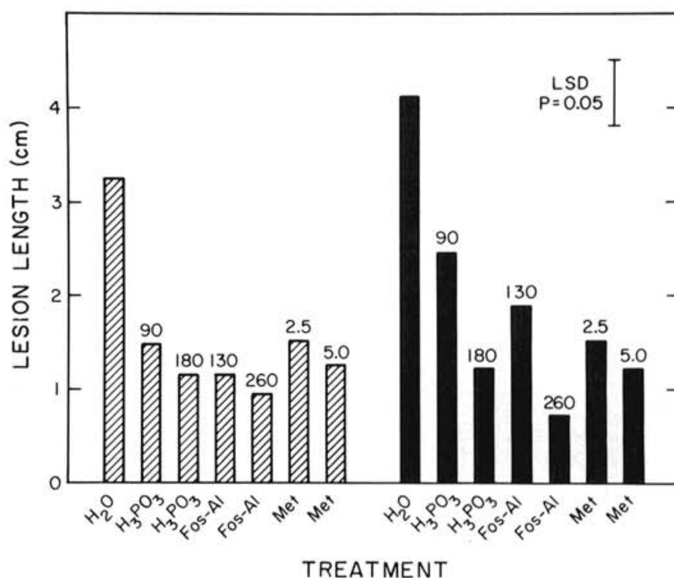

Fig. 1. The effect of phosphorous acid (H_3PO_3), fosetyl-Al (Fos-Al), and metalaxyl (Met) on lesion size in detached tomato leaflets inoculated with *Phytophthora capsici* and floated on various solutions in the presence (solid bars) and absence (striped bars) of 0.08 mM aminooxyacetic acid (AOA). Fungicide concentrations, expressed as micrograms per milliliter, are shown above the histogram bars. Leaflets were detached from greenhouse-grown tomato plants (cultivar Casino Royale), immediately inoculated with an agar disk from a culture of *P. capsici*, and floated on one of the treatment solutions for 3 days.

tolerant isolates of *P. capsici* were identical in sensitivity to metalaxyl in vivo (Table 1).

In vivo effects of AOA and glyphosate on the activity of H_3PO_3, fosetyl-Al, and metalaxyl. The addition of 0.08 mM AOA caused a 28% increase in lesion length, compared to the water control, on tomato leaflets inoculated with *P. capsici* (P1391-1) (Fig. 1). Lesion lengths in the leaflets that received H_3PO_3 at 90 μg/ml and fosetyl-Al at 130 μg/ml were 48 and 62% greater, respectively, when AOA was added (Fig. 1). However, both H_3PO_3 and fosetyl-Al at 180 and 260 μg/ml, respectively, were as effective in reducing lesion size in AOA-treated leaflets as in leaflets not treated with AOA. AOA had no significant effect on the activity of metalaxyl (Fig. 1). Glyphosate did not reverse the in vivo activity of either fosetyl-Al or H_3PO_3 (Table 2).

Uptake of H_3PO_3 by detached tomato leaflets and its relation to lesion restriction. In leaflets floated on H_3PO_3, the concentration of H_3PO_3 near the base of the leaflet was higher than in other parts. In leaflets floated on a solution containing H_3PO_3 at 120 μg/ml, the concentration of H_3PO_3 in the basal one-fourth segment of the leaflet was 245 μg/g fr. wt. of leaflet tissue. The adjacent one-fourth segment of the leaflet contained 195 μg/g, and the apical half of the leaflet contained 101 μg/g fr. wt. To correlate H_3PO_3 concentration with lesion restriction, a parallel experiment was performed wherein the leaflet base of leaflets treated with 120 μg/ml H_3PO_3 was inoculated with P1319-1. Lesion length was reduced by 56%, from 4.1 cm in the control to 1.8 cm in the H_3PO_3-treated leaflets, over 4 days. In another experiment, increasing external concentrations resulted in higher levels of H_3PO_3 in tomato leaflets and a corresponding reduction in lesion length (Table 3).

Comparison of EC_{50} values for H_3PO_3 and levels of phosphate in vivo and in vitro. The in vitro EC_{50} value for *P. capsici* (P1319-1) growing on V8C agar was 80 μg of H_3PO_3 per milliliter. Comparatively, with detached tomato leaflets in vivo, a concentration of 245 μg H_3PO_3/g fr. wt. near the inoculation point reduced lesion expansion by 56%. Because phosphate reduces the antifungal activity of H_3PO_3, the phosphate levels of tomato leaflets and V8C broth were determined. Tomato leaflets contained 634 μg/g fr. wt. inorganic phosphate, while V8C broth contained 188 μg of inorganic phosphate per milliliter.

Effects of AOA on uptake of H_3PO_3 by *P. capsici*. AOA reduced uptake of H_3PO_3 by *P. capsici* in vitro (Tables 4 and 5). Uptake of H_3PO_3 by *P. capsici* was reduced 62 and 74% after 3 hr with 0.08 and 0.25 mM AOA, respectively (Table 4). With isolates P1319-1 and H36, uptake of H_3PO_3 was reduced by 83 and 58%, respectively, in the presence of 0.25 mM AOA (Table 5).

TABLE 2. Interaction and analysis of variance of glyphosate and phosphorous acid (H_3PO_3) in preventing lesion expansion in tomato (cultivar Casino Royale) leaflets inoculated with *Phytophthora capsici* (P1319-1)[x]

H_3PO_3	Glyphosate None	Glyphosate 10 μM	Glyphosate 100 μM	Mean
None	2.7	3.1	phytotoxic[z]	2.9 a[y]
120 μg/ml	0.8	0.6	0.8	0.7 b
Mean	1.75 a[y]	1.9 a		

Source of variation	df	Sum of squares
Treatments	4	29.3
Glyphosate	2	4.4
H_3PO_3	1	24.4
Glyphosate-H_3PO_3 interaction	1	0.5

[x] Detached tomato leaflets were inoculated with 0.4-cm-diameter plugs of *P. capsici* (P1319-1) and floated on the various solutions.
[y] Mean values with the same letter for H_3PO_3 treatment (rows) or glyphosate treatment (columns) are not significantly different according to Duncan's multiple range test ($P = 0.05$).
[z] Phytotoxicity consisting of necrotic spots was observed on the leaves.

TABLE 3. Uptake of phosphorous acid (H_3PO_3) by detached tomato leaflets floating on solutions containing tritiated H_3PO_3

H_3PO_3 concentration of floating solution (μg/ml)	H_3PO_3 concentration in leaflets (μg/g fr. wt.)[x]	Lesion length[y] (cm)
0	–	5.1 a[z]
30	31	4.3 b
60	56	2.8 c
120	131	2.4 cd
180	151	2.1 d

[x] Uninoculated leaflets were floated on water or on a solution containing 30-180 μg/ml H_3PO_3 and 0.038 μCi/ml of a 200 mCi/mmole solution of tritiated H_3PO_3 for 24 hr prior to extraction of H_3PO_3.
[y] Lesion length data are from the same experiment in which, following 24 hr of uptake, leaflets were inoculated with *P. capsici* (P1319-1) and floated on water for 4 days.
[z] Values with the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

DISCUSSION

Neither fosetyl-Al nor H_3PO_3 controlled lesion development in tomato leaflets inoculated with an H_3PO_3-tolerant isolate of *P. capsici*. This provides strong support for a direct mode of action of fosetyl-Al and H_3PO_3.

The concentration of H_3PO_3 in detached leaflets floating on 120 μg/ml of H_3PO_3 was 245 μg/g fr. wt. at the point of inoculation, and lesion size of the wild-type isolate of *P. capsici* was reduced 56%. The H_3PO_3 concentration in the leaflets (245 μg/g fr. wt.) was

TABLE 4. Uptake of phosphorous acid (H_3PO_3) by *Phytophthora capsici* (isolate P1319-1) in liquid culture in the presence and absence of aminooxyacetic acid (AOA)[x]

Time of uptake (hr)	DPM/mg dry wt.[y] no AOA	AOA (0.08 mM)	AOA (0.25 mM)
1	516	427(17)[z]	284(45)
2	1,036	647(38)	388(63)
3	2,204	838(62)	578(74)

[x] Uptake began 12 hr after adding zoospores to liquid medium. Final zoospore concentration equaled 1.0×10^5 zoospores per milliliter. Uptake was started by adding 50 μg/ml unlabeled H_3PO_3 and 0.038 μCi/ml of a 200 mCi/mmole solution of tritiated H_3PO_3 to the fungal culture.
[y] Values are disintegrations per minute per milligram dry weight of *P. capsici* (P1319-1) following incubation with $[^3H]$-H_3PO_3 and washing of fungal suspension with 0.084 mM KH_2PO_4. Dry weight of 10 ml of fungal culture at the time of uptake was 1.87 mg.
[z] Figures in parentheses are percent inhibition of H_3PO_3 uptake compared to the control (no AOA).

TABLE 5. Uptake of phosphorous acid (H_3PO_3) by an H_3PO_3-sensitive (P1319-1) and H_3PO_3-tolerant isolate (H36) of *Phytophthora capsici* in liquid culture in the presence and absence of aminooxyacetic acid (AOA)[x]

Uptake time (hr)	H_3PO_3-sensitive (P1319-1), AOA (mM) 0	0.25	0.5	H_3PO_3-tolerant (H36), AOA (mM) 0	0.25	0.5
	DPM/mg dry wt.[y]					
1	1,145	367(68)[z]	443(61)	596	325(45)	257(57)
2	3,448	579(83)	517(85)	1,066	450(58)	311(71)

[x] Uptake began 11.5 hr after adding zoospores to liquid media. Final zoospore concentration was 2.0×10^5 zoospores per milliliter. Uptake was started by adding unlabeled H_3PO_3 at 50 μg/ml and 0.038 μCi/ml of a 200 mCi/mmole solution of $[^3H]$-H_3PO_3 to the fungal culture.
[y] Values are disintegrations per minute per milligram dry weight of *P. capsici* (P1319-1) following incubation with $[^3H]$-H_3PO_3 and washing of fungal suspension with 0.084 mM KH_2PO_4. Dry weights of 10 ml of the fungal culture of the wild-type (P1319-1) and the H_3PO_3-tolerant isolate (H36) were 2.75 mg and 1.45 mg respectively, at the time of initial uptake.
[z] Figures in parentheses are percent inhibition of H_3PO_3 uptake compared to the control (no AOA).

three times the in vitro EC_{50} concentration for *P. capsici* and would appear to be sufficient to account for inhibition of lesion development due to a direct effect of H_3PO_3 on the pathogen. The phosphate concentration in tomato leaflets was 3.4 times higher than in V8C medium, which could explain why higher concentrations of H_3PO_3 were needed in vivo to inhibit *P. capsici*. Phosphate reduces the antifungal activity of H_3PO_3 and fosetyl-Al both in vitro and in vivo (6,12).

Support for the hypothesis that fosetyl-Al stimulates host defense mechanisms has been provided by the report (5) that AOA and glyphosate reversed the antifungal activity of fosetyl-Al in tomato leaflets infected with *P. capsici*. This interpretation of the results was based on the untested assumption that AOA acted as an inhibitor of the phenylpropanoid pathway in tomato (1,17). However, L-α-aminooxy-β-phenylpropionic acid, which is a highly specific and potent inhibitor of phenylalanine ammonia lyase (1,17), was found (5) to have no effect on the efficacy of fosetyl-Al. In our experiments, glyphosate, an inhibitor of the shikimic acid pathway (2,17,25), did not reverse the antifungal activity of either fosetyl-Al or H_3PO_3. Furthermore, in our experiments, the efficacy of fosetyl-Al (260 μg/ml) at nearly the same concentration used by Bompeix et al (5) (250 μg/ml), was not affected by AOA. We also found that AOA had no effect on lesion size in leaflets treated with 180 μg/ml H_3PO_3. Thus, there now appears to be little credibility for the evidence supporting the hypothesis that the primary mode of action of fosetyl-Al involves the triggering of host defense mechanisms.

The activity of suboptimal concentrations of H_3PO_3 (90 μg/ml) and fosetyl-Al (130 μg/ml) was partially reduced in vivo by AOA. It could be that leaflets treated with AOA were more susceptible to *P. capsici* and therefore higher concentrations of H_3PO_3 and fosetyl-Al were needed to achieve an equivalent level of disease control. However, this seems unlikely since metalaxyl was equally effective in inhibiting lesion expansion with or without AOA treatment. An alternative explanation for the partially inhibitory effects of AOA is that it inhibits H_3PO_3 uptake into the fungus as demonstrated in vitro with *P. capsici*. Presumably, at higher concentrations of H_3PO_3 and fosetyl-Al, sufficient fungicide is taken up by *P. capsici* to inhibit fungal growth despite the partially inhibitory influence of AOA on uptake.

The inhibitory levels of H_3PO_3 found in tomato leaflets floating on H_3PO_3 and the in vivo tolerance of the H36 isolate to both H_3PO_3 fosetyl-Al provide strong evidence that the primary mode of action of H_3PO_3 and fosetyl-Al in the tomato-*P. capsici* system involves a direct inhibition of the pathogen by these toxophores.

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