Disease Control and Pest Management

Quantification of Phosphonate and Ethyl Phosphonate in Tobacco and Tomato Tissues and Significance for the Mode of Action of Two Phosphonate Fungicides

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ABSTRACT

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Quantification of ethyl phosphonate and phosphonate (HPO$_4^{2-}$, phosphite) in plant tissues treated with the phosphonate fungicides fosetyl-Al and potassium phosphonate was achieved using high performance ion chromatography. Phosphonate also was quantified by scintillation counting using tritium-labeled HPO$_4^{2-}$. Lesion length in HPO$_4^{2-}$-treated tomato leaflets inoculated with Phytophthora capsici and containing 8.9 mM phosphate and 78-110 µg/g fresh weight (fr. wt.) of HPO$_4^{2-}$ was reduced by 61%. Tomato leaflets treated with 160 µg/ml of fosetyl-Al for 48 hr contained 88 µg/g fr. wt. of HPO$_4^{2-}$ but only 3 µg/g fr. wt. of ethyl phosphonate. Because the in vitro EC$_50$ value for P. capsici with media containing 5 mM potassium phosphate was 77 µg/ml of H$_2$PO$_4^{2-}$, these combined results support a direct mode of action for HPO$_4^{2-}$. Likewise with P. parasitica var. nicotianae on tobacco, the HPO$_4^{2-}$ content of seedlings treated with 390 µg/ml of HPO$_4^{2-}$ or 1,000 µg/ml of fosetyl-Al (279 µg/g and 308 µg/g, respectively) was sufficient to account for disease control through a direct mode of action. Using chemical mutagenesis, strains of P. capsici and P. parasitica var. nicotianae were obtained which grew on 0.5% cornmeal agar containing 878 µg/ml of HPO$_4^{2-}$. One of the mutant strains of P. parasitica var. nicotianae killed tobacco seedlings containing 484 µg/g fr. wt. of HPO$_4^{2-}$, whereas plants inoculated with the parental wild-type isolate were symptomless in the presence of 215 µg/g fr. wt. of HPO$_4^{2-}$. Uptake of HPO$_4^{2-}$ by P. parasitica var. nicotianae was inhibited 77-80% over 4 hr when a-aminoxyacetic acid (AOA) was added to culture media. In the presence of AOA in vivo, 390 µg/ml of HPO$_4^{2-}$ protected tobacco plants from infection with P. parasitica var. nicotianae, whereas 195 µg/ml was ineffective. These data add further support to the concept that both potassium phosphonate and fosetyl-Al, through the activity of HPO$_4^{2-}$, have a direct mode of action against Phytophthora species in their hosts.

Additional keywords: Aellite, aluminum tris-O-ethyl phosphonate.

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fosetyl-Al or HPO$_4^{2-}$. Levels of HPO$_4^{2-}$ were determined by both high performance ion chromatography (HPIC)(23) and radiolabel methods (17). Further, the performance of HPO$_4^{2-}$-resistant mutants of two species of Phytophthora were assessed in vivo using both potassium phosphate and fosetyl-Al.

MATERIALS AND METHODS

Quantification of HPO$_4^{2-}$ by ion chromatography and radiolabel methods in detached tomato leaflets. Detached tomato leaflets from 8-wk-old plants of the cultivar Bonnie Best were floated adaxial side up on 15 ml of distilled water, HPO$_4^{2-}$ at a range of concentrations, or HPO$_4^{2-}$ to which a constant ratio of [H]$^+$/HPO$_4^{2-}$ was added (ratio equivalent to 0.026 μCi/ml of a 120-mCi/mmol solution for every 59 μg/ml of unlabeled HPO$_4^{2-}$). Tritium-labeled HPO$_4^{2-}$ with tritium bound to the phosphorus atom was synthesized by Amersham, Arlington Heights, IL, as described previously (17). All solutions were titrated to pH 6.2 with KOH. Twenty-four hours after placing on the solutions, the four leaflets were incubated at their base with an HPO$_4^{2-}$-sensitive or -resistant isolate (P1319) or -resistant isolate (P1361) of P. capsici. For 48 hr, leaflets were rinsed with distilled deionized water. Fifty milligrams fresh weight of tissue from the leaflet base was placed in a screw-cap vial, frozen by adding liquid nitrogen, and ground to a powder. Distilled water (3.95 ml) was added, and the vials were incubated at 37°C for 5 min in an Eppendorf microfuge (model 5414) (Fisher Scientific, Pittsburgh, PA). In the case of tritium-labeled samples, 1 ml of the resulting supernatant was suspended in 13 ml of liquid scintillation fluid (Beckman Ready-Solv. HP/b High Performance for aqueous samples, Beckman Instruments, Inc., Fullerton, CA) and counted in a Beckman model 7500 liquid scintillation counter. Counts were converted to micrograms of HPO$_4^{2-}$ taken up on per gram fresh weight of leaflet. The supernatant of unlabeled samples was further prepared for quantification by HPIC via passage through two Ca SEP-PAR cartridges (Waters Associates, Milford, MA) and by filtration with a 0.22-μm membrane filter. Samples were further diluted 10-fold with deionized distilled water before analysis. The HPIC instrument used was a Dionex system 2000i/SP with a model AMMS-1 anion micromembrane suppressor and a conductivity detector (Dionex Corp., Sunnyvale, CA). For analysis of HPO$_4^{2-}$, a separator column HPIC-AS4A was used in conjunction with guard columns MPIC-NGI and HPIC-AG4A (23). The regenerant was used 15 mL H$_2$SO$_4$, and the eluent contained 0.53 mM NaHCO$_3$ plus 1.54 mM Na$_2$CO$_3$. Eluent flow rate was 1.8 ml/min with a system pressure of 10,000 kPa. Flow rate for the regenerant was 2.4 ml/min. The level of quantitative detection for HPO$_4^{2-}$ was 0.1 μg/ml. Phosphate and HPO$_4^{2-}$ standards were used as references in determining the amount of these anions in plant extracts (23). These values were converted to micrograms of HPO$_4^{2-}$ per gram fresh weight of leaflet. The calculated values for HPO$_4^{2-}$ content do not account for HPO$_4^{2-}$ lost in the extraction process and therefore probably underestimate the HPO$_4^{2-}$ content of the leaflets.

Quantification of phosphate compounds in detached tomato leaflets treated with fosetyl-Al. Other leaflets were treated with fosetyl-Al (technical grade, 98% w. v.) and extracted in the same manner as the HPO$_4^{2-}$-treated leaflets. Four replicate leaflets at each concentration of fosetyl-Al were incubated at the leaflet base with a HPO$_4^{2-}$-sensitive (P1319) or -resistant (P1361) isolate as described previously (17). The prepared plant extracts were analyzed for HPO$_4^{2-}$ content by HPIC as described above. Analysis of ethyl phosphate with HPIC, separator column MPIC-AS4A was used in conjunction with guard columns MPIC-NGI and HPIC-AG6. Regenerant was 30 mL H$_2$SO$_4$, and the eluent was 3.5 mM NaOH (23). Eluent flow rate was 1.4 mL/min with a system pressure of 10,000 kPa. Regenerant flow rate was adjusted to 4.5 mL/min. The level of quantitative detection for ethyl phosphate was 0.5 μg/ml. Ethyl phosphate standards were used to quantify levels of ethyl phosphate present. Values were converted to microgram ethyl phosphate per gram fresh weight of leaflet but were not adjusted for extraction efficiency.

Quantification of phosphate compounds in tobacco seedlings. Ethyl phosphate and HPO$_4^{2-}$ were also quantified in 5-wk-old tobacco seedlings, cultivar Burley, which were drenched with fosetyl-Al (technical grade, 98% w. v.) or HPO$_4^{2-}$ for 48 hr. Tobacco seedlings were grown for 4 wk in the greenhouse in UC mix (50% fine sand, 50% peat moss, plus 2.2 kg of dolomite, 1.5 kg of superphosphate, 148 g of KNO$_3$ and 148 g of K$_2$SO$_4$ per cubic meter) (2). Then three seedlings of similar size were transplanted into the center of 355-μl Styrofoam cups with drainage holes and filled with vermiculite. Three to four days after transplanting, the cups were placed in the bottom half of 8.5-cm plastic petri plates in a growth chamber at 26°C under a daily 12-hr photoperiod. Light was from Sylvania GroLux lights (160 W) giving a photon flux density of 170 μE/m$^2$/sec. The vermiculite then was drenched thoroughly with either distilled water, fosetyl-Al, or potassium phosphate. The pH of all solutions was adjusted to 6.2 with KOH. Twenty-four hours after drenching, plants were inoculated by pipetting 10 μl of a suspension of 2×10$^6$ zoospores/ml of P. parasitica var. nicotianae (P1352) down the stems of the three plants per cup. Five days after inoculation, disease was evaluated based on a visual scale where 0 = healthy and 3 = dead.

Stems were cut into pieces 0.4 cm in length, dipped in ethanol, rinsed with distilled water, and blotted and plated onto PPAR medium (21) modified by the substitution of 125 μg/ml of ampicillin (85%), Bristol Laboratories, Syracuse, NY) for 250 μg/ml of sodium ampicillin. After 2 days, the stem pieces from which Phytophthora was recovered were counted and the percentage of stem pieces infected was calculated. There were six replicates per treatment.

Characterization of HPO$_4^{2-}$-sensitive and -resistant isolates of P. capsici and P. parasitica nicotianae. Three fungi were used in mutagenesis experiments: P. capsici (P1503), isolated from tomato in the Ivory Coast and obtained as isolate 375 from G. Bonpeix, Université P. et M. Curie, Pathologie Végétale T53.4, Paris, France; and two isolates of P. parasitica var. nicotianae from tobacco: P1495 from D. I. Guest, School of Botany, University of Melbourne, Parkville, Victoria, Australia, and P1352 from D. H. Shew, Department of Plant Pathology, North Carolina State University, Raleigh. P1503 refers to the isolate numbers for these fungi as maintained under cryogenic storage in the Phytophthora collection at the University of California, Riverside. Strains resistant to HPO$_4^{2-}$ were obtained by treating 0.5×10$^6$ zoospores/ml N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and then overlaying the spores with media containing HPO$_4^{2-}$ as described previously (6).

Varying amounts of HPO$_4^{2-}$ were added to 0.5% Difco cornmeal agar (CMA) containing 1.2% Difco Bacto agar. Parental wide-type isolates of P. capsici (P1503) and P. parasitica var. nicotianae (P1495 and P1352) and their respective HPO$_4^{2-}$-resistant mutants were tested for sensitivity to HPO$_4^{2-}$ by placing a 0.5-cm-diameter agar disk from an actively growing colony on V8C agar fungal side down in the center of each plate. Plates were incubated at 24°C. Radial growth was determined by measuring colony diameter at two points on each petri plate and taking the average value, having subtracted the diameter of the fungal plug. Regression analysis of the results was performed with a computer program using the method of Goldstein (18) for analysis of a graded response. Percent inhibition of radial growth, compared to growth on agar without HPO$_4^{2-}$, was plotted on a probit scale versus log concentration. From this analysis, EC$_{50}$ values with upper and lower confidence limits (P = 0.05) were obtained.

An HPO$_4^{2-}$-resistant isolate (P1755) derived from P. parasitica var. nicotianae (P1352) was tested for its in vivo response to HPO$_4^{2-}$ using 5-wk-old tobacco seedlings of the cultivar Burley. Plants were grown, drenched with HPO$_4^{2-}$, and inoculated as
The concentration of HPO$_2^{-}$ and P, in tobacco stems was quantified by HPIC. Thirty-five milligrams of lower stem tissue was frozen with liquid nitrogen and ground to a powder. This was then extracted in 1.4 ml of deionized distilled water and prepared for analysis as described.

Effects of α-aminoxyacetic acid on disease control by HPO$_2^{-}$ in tobacco seedlings and on fungal uptake of HPO$_2^{-}$. Tobacco plants were grown and treated as described above. The vermiculite in which the tobacco seedlings were growing was drenched with water, potassium phophonate, α-aminoxyacetic acid (AOA), or potassium phophonate plus AOA to study the effects of AOA on the efficacy of HPO$_2^{-}$. Nine seedlings were used for each treatment. Twenty-four hours after drenching, plants were inoculated with zoospores of P. parasitica var. nicotianae (P1352). Three days after inoculation, disease severity was assessed by placing 0.4-cm stem and upper root segments onto PARP-selective media and calculating the percent pieces infected.

To determine the effect of AOA on uptake of HPO$_2^{-}$ by P. parasitica var. nicotianae (P1352), the fungus was incubated at 24°C for 6 days on V8C agar in 8.5-cm-diameter petri plates and then overlaid with 10 ml of sterile 1% soil extract. Plates were incubated at 24°C for another 8 days, and then the soil extract was removed. The plates were rinsed with 10 ml of sterile water, and after an additional 10 ml of water was added, the plates were placed at 4°C for 20 min. Petri plates were subsequently incubated at 24°C for 40 min to allow for sporulation. Uptake of HPO$_2^{-}$ in the presence and absence of AOA was then performed as described previously (17) except that, in addition to scintillation counting of the HPO$_2^{-}$, taken up by the fungus, parallel aliquots were analyzed for HPO$_2^{-}$ uptake by HPIC. Fungal samples were prepared for analysis by filtering 10-ml samples of the fungal suspension through Whatman GF/A filter disks (2.4 cm diameter) (Whatman Inc., Clifton, NJ) and rinsing with four 10-ml aliquots of distilled water. The filter disks with rinsed mycelium were placed on a Braun homogenizer vial (Braun Scientific, Rochester, NY) with 3 ml of distilled water and 2.5 ml of 0.5-mm glass beads and homogenized for 15 sec. The homogenate was centrifuged and the supernatant was filtered through a 0.45-μm membrane filter before analysis by HPIC.

Effect of phosphate on the antifungal activity of HPO$_2^{-}$. Fungi were grown on Ribeiro’s modified synthetic agar medium (RMSM) (16,26) to determine the effects of 5, 10, 15, or 45 mM potassium phosphate buffer on the antifungal activity of HPO$_2^{-}$. RMSM was prepared with 17 g of Difco Bacto agar per 900 ml of water, and the pH was adjusted to 6.2. The appropriate amount of potassium phosphate was added to 100 ml of deionized distilled water, and the pH was adjusted to 6.2 with KOH. After autoclaving, the potassium phosphate solution, thiamine HCl (1 mg/L), and filter-sterilized potassium phosphate (pH 6.2) were added to the medium, and 15 ml was dispensed into 8.5-cm-diameter plastic petri plates. A 0.25-cm-diameter agar disk, from an actively growing colony on V8C agar, was placed with the fungal side down in the center of each plate. Plates were incubated at 24°C in the dark. Radial growth was determined by measuring colony diameter at two points on each petri plate and taking the average value, having subtracted the diameter of the fungal plug. Percentage inhibition was based on colony growth on agar without HPO$_2^{-}$. Regression analysis plotting percent inhibition on a probit scale versus log concentration was performed as described above.

RESULTS

Quantification of HPO$_2^{-}$ in detached tomato leaflets by ion chromatography and scintillation counting. No statistical differences were found in the levels of HPO$_2^{-}$ in tomato extracts using radiolabel counting and HPIC (Table 1). In most cases the HPO$_2^{-}$ content of the leaflets increased as its concentration was raised in the floating solution. With increasing HPO$_2^{-}$ concentration in the leaflets, there was a corresponding reduction in the size of disease lesions on leaflets inoculated with a HPO$_2^{-}$-sensitive isolate of P. capsici (P1319). The response of the HPO$_2^{-}$-resistant strain of P. capsici to increasing levels of HPO$_2^{-}$ in plant tissue was much less marked (Table 1).

When standard solutions of potassium phosphonate containing [H]-HPO$_2^{-}$ were fractionated by HPIC, 94-95% of the tritium was in the HPO$_2^{-}$ peak. Even after 8 mo of storage under nitrogen gas at 5°C (pH = 6.5), 95% of the total radioactivity of [H]-HPO$_2^{-}$ was in the HPO$_2^{-}$ peak fraction obtained by HPIC. When extracts of...
tomato leaflets treated with [3H]-PO₂⁻ for 24 hr were fractionated by HPIC, 93% of the tritium label was found in the HPO₂⁻ fraction.

Quantification of phosphonate anions in detached tomato leaflets and tobacco seedlings treated with fosetyl-Al. Tomato leaflets treated with 400 μg/ml of fosetyl-Al contained only 14 μg/g fresh weight (fr. wt.) of ethyl phosphonate anion 48 hr after treatment (Table 2). No ethyl phosphonate was detected in tobacco seedlings 4 days after treatment with fosetyl-Al (Table 2).

Increasing levels of HPO₂⁻ were found in the tomato leaflets and tobacco seedlings as the concentration of fosetyl-Al in the treatment solutions was increased (Tables 2 and 3). When the concentrations of the floating solutions ranged from 80 to 400 μg/ml of fosetyl-Al, the corresponding range of HPO₂⁻ levels was 49–358 μg/g fr. wt. in tomato leaflets (Table 2). In the tobacco seedling experiment, the concentration range for drench solutions of 250–1,500 μg/ml of fosetyl-Al resulted in HPO₂⁻ concentrations of 81–316 μg/g fr. wt. in tobacco tissue consisting of lower stems and upper tap root segments (Table 3).

Characterization of HPO₂⁻ sensitive and -resistant strains of P. capsici and P. p. nicotianae. Strains that grew on 0.5% CMA in the presence of 878 μg/ml of potassium phosphonate (pH 6.2) were readily obtained by chemical mutagenesis of P. capsici (isolate P1503) and P. parasitica var. nicotianae (isolates P1352 and P1495). The parental isolates had EC₀ values ranging from less than 49–57 μg/ml, whereas the EC₀ values for the corresponding resistant strains ranged from 270 to 815 μg/ml (Table 4).

An HPO₂⁻ resistant mutant (P1753) of P. parasitica var. nicotianae (P1352) had an in vitro EC₅₀ value of 665 μg/ml and was evaluated for in vivo resistance by comparing the incidence of disease in tobacco seedlings treated with 548 HPO₂⁻ and inoculated with either P1352 or the HPO₂⁻ resistant mutant (P1755). In tobacco plants inoculated with P1352 and treated with 195 μg/ml of HPO₂⁻, visual symptoms were slight although 60% of stem pieces were actually infected, as determined by plating out stem segments on PARP selective medium. However, in plants treated with 390 μg/ml of HPO₂⁻, only 4% of stem pieces were infected (Table 5). In contrast, in plants inoculated with the mutant (P1755), virtually all plants died at every HPO₂⁻ concentration except the highest (1,560 μg/ml), in which case plants were symptomless even though 38% of stem pieces were actually infected with P. parasitica var. nicotianae (Table 5).

The HPO₂⁻ concentration in stems of treated tobacco plants increased in relation to the concentration of the drench solution.
The treatments ranged from 195 to 1,560 μg/ml of HPO$_4^{2-}$, whereas the corresponding concentrations measured by HPIC in the tobacco stem were 215–755 μg/g fr. wt. (Table 5). The P concentration in the stems was 3.2 mM as determined by HPIC.

Comparison of in vivo and in vitro antifungal activity of phosphonate. The concentration of HPO$_4^{2-}$ in culture media and in tomato and tobacco tissue, along with the corresponding levels of inhibition of P. capsici or P. parasitica var. nicotianae are presented in Table 6. The HPO$_4^{2-}$-sensitive isolates were similarly inhibited by HPO$_4^{2-}$ in vitro and in vivo. On the other hand, the resistant isolates were only partially inhibited by high concentrations of HPO$_4^{2-}$ in culture media or in plant tissue (Table 6).

Effect of AOA on disease control by HPO$_4^{2-}$ in tobacco seedlings and on fungal uptake of HPO$_4^{2-}$. Treatment of tobacco seedlings with 0.08 mM AOA increased the percentage of stem pieces infected with P. parasitica var. nicotianae (P1352) from 43% infected in the water control to 75% in the AOA treatment (Fig. 1). HPO$_4^{2-}$ at 195 μg/ml reduced stem infection to 9%. However, in the presence of 0.08 mM AOA and 195 μg/ml of HPO$_4^{2-}$, 63% of the stem pieces were infected. In tobacco seedlings treated with 390 μg/ml of HPO$_4^{2-}, 7$ and 10% of the stem pieces were infected in the absence and presence of AOA, respectively (Fig. 1).

The in vitro effect of 0.08 mM AOA on uptake of HPO$_4^{2-}$ by P. parasitica var. nicotianae was determined by both HPIC and scintillation counting. After 4 hr in the presence of 98 μg/ml of HPO$_4^{2-}$, the HPO$_4^{2-}$ concentration in the mycelium was 2.03 and 2.00 μg/ml fr. wt. as determined by HPIC and radioisotope methods, respectively. In the presence of 0.5 mM AOA, uptake of HPO$_4^{2-}$ was inhibited 77–80% after 4 hr as determined by both methods. There were no significant differences in HPO$_4^{2-}$ quantification between the two methods.

Effects of phosphate level on the antifungal activity of HPO$_4^{2-}$. An HPO$_4^{2-}$-sensitive isolate (P1319) and a resistant mutant (P1361) of P. capsici and isolect P1352 of P. parasitica var. nicotianae were grown on RMSM with 5, 10, 15, or 45 mM potassium phosphate (P) using a range of HPO$_4^{2-}$ concentrations. With P. capsici (P1319), HPO$_4^{2-}$ was more inhibitory to mycelial growth in the presence of 5 mM P than in the presence of 15 or 45 mM P (Fig. 2). The respective EC$_{50}$ values at 5, 10, 15, and 45 mM P were 77, 135, 184, and 186 μg/ml of HPO$_4^{2-}$. The HPO$_4^{2-}$-resistant mutant of P. capsici (P1361) also was more inhibited with 5 mM P than with 15 or 45 mM P (Fig. 3). The EC$_{50}$ values at 5, 15, and 45 mM P were 344, 607, and 503 μg/ml of HPO$_4^{2-}$, respectively. Each EC$_{50}$ value was statistically different from the other two values (P = 0.05).

Increasing the P concentration from 5 to 15 mM reduced the antifungal activity of HPO$_4^{2-}$ toward P. parasitica var. nicotianae (P1352) (Fig. 4). The respective EC$_{50}$ values with 5 and 15 mM P were 55 and 123 μg/ml of HPO$_4^{2-}$. However, further increasing the P content of the media from 15 to 45 mM resulted in an EC$_{50}$ value of 78 μg/ml, which was not significantly different from the EC$_{50}$ values for HPO$_4^{2-}$ at 5 and 15 mM.

**DISCUSSION**

Tomato leaflets containing 88 μg/ml of HPO$_4^{2-}$ and tobacco stems containing 215 μg/ml of HPO$_4^{2-}$ did not develop symptoms when inoculated with HPO$_4^{2-}$-sensitive isolates of P. capsici or P. parasitica var. nicotianae. On the other hand, HPO$_4^{2-}$-resistant isolates of P. capsici and P. parasitica var. nicotianae were pathogenic on tomato leaflets or tobacco stems that contained 554 or 484 μg/g fr. wt. of HPO$_4^{2-}$. The most obvious interpretation of these results is that HPO$_4^{2-}$ controls plant disease by directly inhibiting the pathogen.

The extremely low concentration or total absence of ethyl phosphonate and the high levels of HPO$_4^{2-}$ in tomato leaflets and tobacco seedlings treated with fosetyl-Al agrees with previous reports suggesting a rapid degradation of fosetyl-Al to HPO$_4^{2-}$ in plant tissue (7,11,22,23,27). This strengthens the concept that

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**TABLE 6.** Comparison of in vivo and in vitro antifungal activity of phosphonate (HPO$_4^{2-}$) toward growth of HPO$_4^{2-}$-sensitive and -resistant isolates of Phytophthora capsici and P. parasitica var. nicotianae.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth medium</th>
<th>HPO$_4^{2-}$ concentration</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. capsici (P1319)</td>
<td>Tomato leaflet, RMSM</td>
<td>78–100$^a$</td>
<td>61</td>
</tr>
<tr>
<td>P. capsici (P1361)</td>
<td>Tomato leaflet, RMSM</td>
<td>484–642$^a$</td>
<td>37</td>
</tr>
<tr>
<td>P. parasitica var. nicotianae (P1352)</td>
<td>Tobacco stem, RMSM</td>
<td>344</td>
<td>50</td>
</tr>
<tr>
<td>P. parasitica var. nicotianae (P1755)</td>
<td>Tobacco stem, RMSM</td>
<td>279</td>
<td>96</td>
</tr>
</tbody>
</table>

$^a$P1319 and P1352 are HPO$_4^{2-}$-sensitive parental isolates. P1361 and P1755 are HPO$_4^{2-}$-resistant mutants obtained by treating zoospores of the parental isolates with the chemical mutagen N-methyl-N-nitro-N-nitrosoguanidine.

$^b$The in vitro antifungal activity of HPO$_4^{2-}$ was determined by measuring radial growth on Ribeiro's modified synthetic medium (RMSM) containing 5 mM P or on 0.5% cornmeal agar (CMA) at various concentrations of HPO$_4^{2-}$.

$^c$Concentrations of HPO$_4^{2-}$ were determined by high performance ion chromatography (HPIC) and are expressed as micrograms per gram fresh weight plant tissue or as micrograms per ml of culture media. Inorganic phosphate concentrations quantified by HPIC were 8.9 mM for tomato leaflets and 3.2 mM for tobacco stems.

$^d$For tomato leaflets, percent inhibition refers to reduction in lesion length in HPO$_4^{2-}$-treated leaflets inoculated with P. capsici. In the case of tobacco stems, values represent inhibition of infection of stem pieces inoculated with P. parasitica var. nicotianae and treated with potassium phosphate. Values for growth on culture media represent the level of inhibition of radial growth at the stated concentrations of HPO$_4^{2-}$.

$^e$The first value was determined using HPIC and the second value was determined from scintillation counting of tritium-labeled HPO$_4^{2-}$ in leaflet extracts.

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**Fig. 1.** Effect of 0.08 mM aminoxyacetic acid (AOA) and/or phosphonate on disease control in tobacco seedlings inoculated with zoospores of Phytophthora parasitica var. nicotianae isolate P1352. The AOA concentration in all treatments with AOA was 0.08 mM. The concentration of phosphonate (HPO$_4^{2-}$) used, expressed in micrograms per milliliter, is shown above the histogram bars. The 95% confidence intervals are shown for each treatment.

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**Fig. 2.** Effect of 0.08 mM aminoxyacetic acid (AOA) and/or phosphonate on disease control in tobacco seedlings inoculated with zoospores of Phytophthora parasitica var. nicotianae isolate P1352. The AOA concentration in all treatments with AOA was 0.08 mM. The concentration of phosphonate (HPO$_4^{2-}$) used, expressed in micrograms per milliliter, is shown above the histogram bars. The 95% confidence intervals are shown for each treatment.
HPO$_4^{2-}$ is the major toxophore responsible for disease control in plants treated with fosetyl-Al (5,7,11,16,17,22,23,25,27).

The amount of HPO$_4^{2-}$ measured in plant tissues and fungal mycelium by both HPIC and radioanalytical techniques was found to be equivalent in all treatments, demonstrating the validity of both methods. Although the tritium label is presumably covalently bound to the phosphorous atom (8–10,15,17) in [H]HPO$_4^{2-}$, the stability of the tritium label had not been previously demonstrated. For this reason, buffered solutions (pH 6.2) of [H]HPO$_4^{2-}$ and extracts of plants treated with [H]HPO$_4^{2-}$ were fractionated by HPIC. The association of 93–95% of the radioactivity with the HPO$_4^{2-}$ peak fraction even after 8 mo of storage as potassium phosphate at pH 6.2 provides evidence that the tritium label is bound to the phosphorus atom in [H]HPO$_4^{2-}$.

There is now a report that a naturally occurring fosetyl-Al-resistant isolate of P. cinnamomi causes disease in Chamaecyparis lawsoniana. Disease control with fosetyl-Al was lost after several years of continuous application. The isolate of P. cinnamomi also was resistant to HPO$_4^{2-}$ in vitro (28). Although the mode of action of HPO$_4^{2-}$ is still unknown, the ability to obtain HPO$_4^{2-}$-resistant strains by either chemical mutagenesis treatment or by natural selection is typical of a fungicidal compound having a site-specific direct mode of action (12,13).

Previously, the efficacy of fosetyl-Al was reported to be reversed by treatment with AOA. This was presented as evidence supporting the hypothesis that fosetyl-Al stimulates host defense mechanisms (3). This interpretation was based on the assumption that AOA is acting through its effects as an inhibitor of the phenylpropanoid pathway in tomato (1,20). However, Fenn and Coffey (17) found that the efficacy of HPO$_4^{2-}$ and ethyl phosphate was only partially reduced by AOA in tomato leaflets inoculated with P. capsici. Uptake of HPO$_4^{2-}$ by P. capsici in vitro was strongly reduced by addition of AOA to the culture medium (17). In this paper similar results are reported with P. parasitica var. nicotianae and tobacco. In vitro uptake of HPO$_4^{2-}$ by a sensitive isolate (P1352) was inhibited 77–80% after 4 hr in the presence of 0.5 mM AOA. In tobacco treated with AOA, 195 µg/ml of HPO$_4^{2-}$ was ineffective in preventing stem infection by P. parasitica var. nicotianae, whereas 390 µg/ml of HPO$_4^{2-}$ resulted in disease control. Thus, in tomato and tobacco, AOA only reversed the antifungal activity of HPO$_4^{2-}$ when relatively low concentrations were applied. Lesion size on potato leaflets inoculated with P. capsici (17) and tobacco stems inoculated with P. parasitica var. nicotianae was significantly greater with AOA treatment compared with the water control. The increase in plant susceptibility caused by AOA combined with the inhibitory effect of AOA on HPO$_4^{2-}$ uptake by the pathogen can explain the need for higher levels of HPO$_4^{2-}$ for disease control in AOA-treated plants.

In some cases a high level of P$_4$ has been shown to reduce the in vitro antifungal activity of HPO$_4^{2-}$ and ethyl phosphate (5,16). Reduction in efficacy with high P$_4$ was far greater with ethyl phosphate than with HPO$_4^{2-}$ (16). In the current study, the HPO$_4^{2-}$-sensitive and -resistant strains of P. capsici and a sensitive isolate of P. parasitica var. nicotianae were less inhibited by HPO$_4^{2-}$ as the P$_4$ level increased from 5 to 15 mM. However, at some concentrations of HPO$_4^{2-}$, inhibition of mycelial growth in vitro was greater at 45 mM P$_4$ than at 15 mM with both P. capsici and P. parasitica var. nicotianae. Dolan and Coffey (14) also found that disease control for P. palmivora on tomato seedlings treated with potassium phosphate was enhanced when higher concentrations of P$_4$ were included in the treatments. Bompeix and Saindrenan (5) found that Phytophthora species varied in the

![Fig 2. Dosage response curve for Phytophthora capsici, isolate P1319, on Ribeiro’s synthetic medium containing different concentrations of phosphate (µg/ml) and either 5, 15, or 45 mM potassium phosphate. Correlation coefficients (r) ranged from 0.96 to 0.99 and all were significantly positive (P = 0.05).](image-url)

![Fig 3. Dosage response curve for a phosphate (HPO$_4^{2-}$)-resistant mutant (P1361) of Phytophthora capsici (P1319) on Ribeiro’s synthetic medium containing different concentrations of HPO$_4^{2-}$ (µg/ml) and either 5, 15, or 45 mM potassium phosphate. Correlation coefficients (r) ranged from 0.93 to 0.97 and all were significantly positive (P = 0.05).](image-url)
degree to which P reduced the antifungal activity of HPO$_4^{2-}$ toward them. They reported that P had little or no impact on the efficacy of HPO$_4^{2-}$ toward *P. palmivora*, a slight effect with *P. parasitica*, and a much stronger effect with *P. capsici* (5). The potentially antagonistic effects of P, concentration on the efficacy of phosphate fungicides probably have been overemphasized. For instance, P concentrations in plant tissues used in this study ranged from 2 to 9 mM. The concentrations of HPO$_4^{2-}$ found in tobacco stems and tomato leaflets (49–753 µg/g fr. wt.) would be sufficient to cause a direct inhibition of the mycelial growth of *P. parasitica var. nicotianae* and *P. capsici*, respectively.

In conclusion, the quantification of inhibitory levels of HPO$_4^{2-}$ in tobacco and tomato tissues treated with fosetyl-Al or potassium phosphate suggests that HPO$_4^{2-}$ is the active principle in preventing infection with *P. capsici* and *P. parasitica var. nicotianae*. The inefficacy of fosetyl-Al and HPO$_4^{2-}$ for disease control in plants inoculated with HPO$_4^{2-}$-resistant mutants provides strong evidence for a direct antifungal mode of action for phosphonate fungicides.

**LITERATURE CITED**


