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_3^{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_3^{2-}. Lesion length in HPO_3^{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_3^{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_3^{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 $H_3PO_3^{-2}$, these combined results support a direct mode of action for HPO_3^{2-}. Likewise with *P. parasitica* var. *nicotianae* on tobacco, the HPO_3^{2-} content of seedlings treated with 390 μg/ml of HPO_3^{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_3^{2-}. One of the mutant strains of *P. parasitica* var. *nicotianae* killed tobacco seedlings containing 484 μg/g fr. wt. of HPO_3^{2-}, whereas plants inoculated with the parental wild-type isolate were symptomless in the presence of 215 μg/g fr. wt. of HPO_3^{2-}. Uptake of HPO_3^{2-} by *P. parasitica* var. *nicotianae* was inhibited 77–80% over 4 hr when α-aminooxyacetic acid (AOA) was added to culture media. In the presence of AOA in vivo, 390 μg/ml of HPO_3^{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_3^{2-}, have a direct mode of action against *Phytophthora* species in their hosts.

Additional keywords: Aliette, aluminum tris-*O*-ethyl phosphonate.

Fosetyl-Al (aluminum tris-*O*-ethyl phosphonate, Aliette, Rhône-Poulenc Agrochimie, Lyon, France) is unique among fungicides in that it is systemic in both a basipetal and acropetal direction (7). Fosetyl-Al, which has poor antifungal activity in vitro, degrades to phosphonate (phosphite, HPO_3^{2-}) in plants and soils (7,11,22,23,25,27). Until recently, researchers were not aware of the direct antifungal activity of HPO_3^{2-} toward mycelial growth of members of the Peronosporales. Therefore, studies were oriented toward the possibility that fosetyl-Al might stimulate host defense mechanisms (3,4,19,29). However, now several reports from different laboratories attest to the important antifungal properties of HPO_3^{2-} (5,7,11,16,17,24,28).

There is insufficient data relating to the levels of phosphonate anions in plant tissue following treatment with either fosetyl-Al or potassium phosphonate. In addition, the concentration of inorganic phosphate (P_i) in culture media has been shown to interfere, in some cases, with the efficacy of fosetyl-Al and to a much lesser degree HPO_3^{2-} (5,16). In this paper we report the levels of HPO_3^{2-} and P_i in tomato and tobacco tissues treated with either

fosetyl-Al or HPO_3^{2-}. Levels of HPO_3^{2-} were determined by both high performance ion chromatography (HPIC) (23) and radiolabel methods (17). Further, the performance of HPO_3^{2-}-resistant mutants of two species of *Phytophthora* were assessed in vivo using both potassium phosphonate and fosetyl-Al.

MATERIALS AND METHODS

Quantification of HPO_3^{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_3^{2-} at a range of concentrations, or HPO_3^{2-} to which a constant ratio of [^{3}H]-PO_3^{-2} was added (ratio equivalent to 0.026 μCi/ml of a 120-mCi/mmole solution for every 59 μg/ml of unlabeled HPO_3^{2-}). Tritium-labeled HPO_3^{2-} with tritium bound to the phosphorous 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, four leaflets were inoculated at their base with an HPO_3^{2-}-sensitive (P1319) or -resistant isolate (P1361) of *P. capsici*.

After 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 laid on their sides and agitated for 1 hr using a reciprocal shaker at 180 strokes (3.5 cm)/min. A 1.5-ml aliquot of each vial was then centrifuged for 5 min in an Eppendorf microfuge (model 5414) (Fisher Scientific, Pittsburg, 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_3^{-2} taken up per gram fresh weight of leaflet. The supernatant of unlabeled samples was further prepared for quantification by HPIC via passage through two C_{18} SEP-PAK 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_3^{2-}, a separator column HPIC-AS4A was used in conjunction with guard columns MPIC-NG1 and HPIC-AG4A (23). The regenerant used was 15 mN H_2SO_4, and the eluent contained 0.53 mM $NaHCO_3$ plus 1.54 mM Na_2CO_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_3^{2-} was 0.1 μg/ml. Phosphate and HPO_3^{2-} standards were used as references in determining the amounts of these anions in plant extracts (23). These values were converted to micrograms of HPO_3^{2-} per gram fresh weight of leaflet. The calculated values for HPO_3^{2-} content do not account for HPO_3^{2-} lost in the extraction process and therefore probably underestimate the HPO_3^{2-} content of the leaflets.

Extracts of tomato leaflets treated with a solution containing HPO_3^{2-} and [^{3}H]-PO_3^{2-} also were analyzed by HPIC. Fractions were collected in 15-ml scintillation vials to determine what percentage of the tritium label was associated with the HPO_3^{2-} peak fraction. To all the collected fractions 13 ml of scintillation fluid was added and samples were counted as already described.

Quantification of phosphonate compounds in detached tomato leaflets treated with fosetyl-Al. Other leaflets were treated with fosetyl-Al (technical grade, 98% a. i.) and extracted in the same manner as the HPO_3^{2-}-treated leaflets. Four replicate leaflets at each concentration of fosetyl-Al were inoculated at the leaflet base with a HPO_3^{2-}-sensitive (P1319) or -resistant (P1361) isolate as described previously (17). The prepared plant extracts were analyzed for HPO_3^{2-} content by HPIC as already described. For analysis of ethyl phosphonate with HPIC, separator column HPIC-AS6 was used in conjunction with guard columns MPIC-NG1 and HPIC-AG6. Regenerant was 30 mN H_2SO_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 phosphonate was 0.5 μg/ml. Ethyl phosphonate standards were used to quantify levels in plant extracts. Values were converted to microgram ethyl phosphonate per gram fresh weight of leaflet but were not adjusted for extraction efficiency.

Quantification of phosphonate compounds in tobacco seedlings. Ethyl phosphonate and HPO_3^{2-} also were quantified in 5-wk-old tobacco seedlings, cultivar Burley, which were drenched with fosetyl-Al (technical grade, 98% a. i.) or HPO_3^{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 dolmite, 1.5 kg of superphosphate, 148 g of KNO_3 and 148 g of K_2SO_4 per cubic meter) (2). Then three seedlings of similar size were transplanted into the center of 355-ml 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 photoflux density of 170 μE/m^2/sec. The vermiculite then was drenched thoroughly with either distilled water, fosetyl-Al, or potassium phosphonate. The pH of all solutions was adjusted to 6.2 with KOH. Twenty-four hours after drenching, plants were inoculated by pipetting 10 ml of a suspension of 2×10^4 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 PARP medium (21) modified by the substitution of 125 μg/ml of ampicillin trihydrate (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_3^{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. Bompeix, 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. P1352, P1495, and P1503 refer 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_3^{2-} were obtained by treating encysted zoospores with 30 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and then overlaying the spores with media containing HPO_3^{2-} as described previously (6).

Varying amounts of HPO_3^{2-} were added to 0.5% Difco cornmeal agar (CMA) containing 1.2% Difco Bacto agar. Parental wild-type isolates of *P. capsici* (P1503) and *P. parasitica* var. *nicotianae* (P1495 and P1352) and their respective HPO_3^{2-}-resistant mutants were tested for sensitivity to HPO_3^{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_3^{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_3^{2-}-resistant isolate (P1755) derived from *P. parasitica* var. *nicotianae* (P1352) was tested for its in vivo response to HPO_3^{2-} using 5-wk-old tobacco seedlings of the cultivar Burley. Plants were grown, drenched with HPO_3^{2-}, and inoculated as

described above.

The concentration of HPO_3^{2-} and P_i 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-aminooxyacetic acid on disease control by HPO_3^{2-} in tobacco seedlings and on fungal uptake of HPO_3^{2-}. Tobacco plants were grown and treated as described above. The vermiculite in which the tobacco seedlings were growing was drenched with water, potassium phosphonate, α-aminooxyacetic acid (AOA), or potassium phosphonate plus AOA to study the effects of AOA on the efficacy of HPO_3^{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 plating 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_3^{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 zoospore release. Uptake of HPO_3^{2-} in the presence and absence of AOA was then performed as described previously (17) except that, in addition to scintillation counting of the $[^3H]$-PO_3^{2-} taken up by the fungus, parallel aliquots were analyzed for HPO_3^{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 disk with rinsed mycelium was placed on a Braun homogenizer vial (Bronwill Scientific, Rochester, NY) with 3 ml of deionized 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 C_{18} SEP-PAK cartridge and a 0.22-μm membrane filter before analysis by HPIC.

Effect of phosphate on the antifungal activity of HPO_3^{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_3^{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 10N KOH. After autoclaving, the potassium phosphate solution, thiamine HCl (1 mg/L), and filter-sterilized potassium phosphonate (pH 6.2) were added to the medium, and 15 ml was dispensed into 8.5-cm-diameter plastic petri plates. A 0.5-cm-diameter agar disk, taken 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_3^{2-}. Regression analysis plotting percent inhibition on a probit scale versus log concentration was performed as described above.

RESULTS

Quantification of HPO_3^{2-} in detached tomato leaflets by ion chromatography and scintillation counting. No statistical differences were found in the levels of HPO_3^{2-} in tomato extracts using radiolabel counting and HPIC (Table 1). In most cases the HPO_3^{2-} content of the leaflets increased as its concentration was raised in the floating solution. With increasing HPO_3^{2-} concentration in the leaflets, there was a corresponding reduction in the size of disease lesions on leaflets inoculated with a HPO_3^{2-}-sensitive isolate of *P. capsici* (P1319). The response of the HPO_3^{2-}-resistant strain of *P. capsici* to increasing levels of HPO_3^{2-} in plant tissue was much less marked (Table 1).

When standard solutions of potassium phosphonate containing $[^3H]$-PO_3^{2-} were fractionated by HPIC, 94–95% of the tritium was in the HPO_3^{2-} peak. Even after 8 mo of storage under nitrogen gas at 5 C (pH = 6.5), 95% of the total radioactivity of $[^3H]$-PO_3^{2-} was in the HPO_3^{2-} peak fraction obtained by HPIC. When extracts of

TABLE 1. Phosphonate (HPO_3^{2-}) concentration of detached tomato leaflets floating on solutions of potassium phosphonate as determined by high performance ion chromatography (HPIC) and use of tritiated HPO_3^{2-}, and disease caused by sensitive and resistant strains of *Phytophthora capsici*[a]

HPO_3^{2-} concentration of floating solution (μg/ml)	HPO_3^{2-} concentration in leaflets (μg/g fresh weight)[b]		Lesion length (cm)[c]	
	HPIC[d]	$[^3H]$-PO_3^{2-}[d]	P1319 sensitive	P1361 resistant
0	0	0	4.1	4.6
59	78	110	1.6	4.8
117	276	344	1.2	3.9
176	320	307	0.9	3.9
234	554	625	0.4	4.1
293	484	642	0.0	2.9

[a] Data are from the same experiment as Table 2.
[b] Uninoculated leaflets were floated on water, a solution of HPO_3^{2-}, or a solution containing 59–293 μg/ml of HPO_3^{2-} and tritiated HPO_3^{2-} in a ratio of 0.026 μCi/ml of a 120 mCi/mmole solution for every 59 μg/ml of unlabeled HPO_3^{2-}. The pH of all phosphonate solutions was adjusted to 6.2 by addition of KOH. Leaflets were floated 48 hr before extraction. Phosphate content of leaflets was 8.9 mM as determined by HPIC.
[c] Following 24 hr of HPO_3^{2-} uptake, a parallel set of leaflets was inoculated with *P. capsici* and floated on the solutions for 4 additional days.
[d] Data from HPIC and radiolabel methods were not significantly different at each concentration of HPO_3^{2-} as tested by a one-way analysis of variance.

TABLE 2. Phosphonate (HPO_3^{2-}) and ethyl phosphonate concentration of detached tomato leaflets floating on solutions containing fosetyl-Al as determined by high performance ion chromatography (HPIC), and disease caused by sensitive and resistant strains of *Phytophthora capsici*[a]

Fosetyl-Al concentration of floating solution (μg/ml)	HPO_3^{2-} concentration (μg/g fresh weight)[b]	Ethyl phosphonate concentration (μg/g fresh weight)[b]	Lesion length (cm)[c]	
			P1319 sensitive	P136 resistant
0	0	0	4.1	4.6
80	49	0.5	1.4	4.2
160	88	3.0	0.4	4.4
240	181	4.0	0.4	3.5
320	222	10.0	0.5	3.6
400	358	14.0	0.4	3.6

[a] Data are from the same experiment as Table 1. Phosphate content of leaflets was 8.9 mM as determined by HPIC.
[b] Uninoculated leaflets were floated on water or a solution containing 80–400 μg/ml of fosetyl-Al for 48 hr before extraction of HPO_3^{2-} and ethyl phosphonate.
[c] Following 24 hr of uptake, a parallel set of leaflets was inoculated with *P. capsici* and floated on the solutions for 4 additional days.

tomato leaflets treated with [^{3}H]-PO_3^{2-} for 24 hr were fractionated by HPIC, 93% of the tritium label was found in the HPO_3^{2-} 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 phosophonate was detected in tobacco seedlings 4 days after treatment with fosetyl-Al (Table 3). Increasing levels of HPO_3^{2-} 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 HPO_3^{2-} 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 HPO_3^{2-} concentrations of 81–316 μg/g fr. wt. in tobacco tissue consisting of lower stems and upper tap root segments (Table 3).

Characterization of HPO_3^{2-}-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 EC_{50} values ranging from less than 49–57 μg/ml, whereas the EC_{50} values for the corresponding resistant strains ranged from 270 to 815 μg/ml of HPO_3^{2-} (Table 4).

An HPO_3^{2-}-resistant mutant (P1755) of *P. parasitica* var. *nicotianae* (P1352) had an in vitro EC_{50} value of 665 μg/ml and was evaluated for in vivo resistance by comparing the incidence of disease in tobacco seedlings treated with HPO_3^{2-} and inoculated with either P1352 or the HPO_3^{2-}-resistant mutant (P1755). In tobacco plants inoculated with P1352 and treated with 195 μg/ml of HPO_3^{2-}, 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 HPO_3^{2-}, only 4% of stem pieces were infected (Table 5). In contrast, in plants inoculated with the mutant (P1755), virtually all plants died at every HPO_3^{2-} 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 HPO_3^{2-} concentration in stems of treated tobacco plants increased in relation to the concentration of the drench solution.

TABLE 3. Phosphonate (HPO_3^{2-}) concentration in stems of 5-wk-old tobacco seedlings drenched with fosetyl-Al as determined by high performance ion chromatography (HPIC) and incidence of disease following inoculation with *Phytophthora parasitica* var. *nicotianae* (P1352)

Fosetyl-Al concentration of drench solution (μg/ml)[a]	HPO_3^{2-} concentration in stem (μg/g fresh weight)[b]	Percent stem pieces infected[c]
0	0	43
250	81	26
500	119	19
1000	308	4
1500	316	0

[a] Twenty-four hours after drenching, plants were inoculated with zoospores of *P. parasitica* var. *nicotianae* (P1352). Four days after drenching, uninoculated plants were extracted for analysis of HPO_3^{2-} and ethyl phosphonate.

[b] Determined by HPIC following water extraction of tobacco stems previously frozen with liquid nitrogen and ground to a powder. No ethyl phosphonate was detected by HPIC in any of the treatments. Phosphate content of stem tissue was 2.3 mM.

[c] Percent stem pieces infected was calculated from the number of stem sections (0.4 cm in length) from which *P. parasitica* var. *nicotianae* was recovered following plating out of stem pieces on selective media. $P = 0.001$ with $X^2 = 44$ for comparison of number of stem pieces infected at each concentration of fosetyl-Al.

TABLE 4. EC_{50} values and regression equations for phosphonate (HPO_3^{2-}) -sensitive and -resistant strains of *Phytophthora capsici* and *P. parasitica* var. *nicotianae* grown on 0.5% cornmeal agar containing potassium phosphonate

Isolate[a]	EC_{50}[b] (μg/ml HPO_3^{2-})	Regression equation[b]
P. capsici (P1503)	57 (54–60)	$y = 2.08x + 1.31$
mutant 2 (P1764)	815 (723–943)	$y = 0.76x + 2.79$
mutant 9 (P1765)	311 (281–347)	$y = 1.61x + 0.98$
mutant 10 (P1766)	462 (408–545)	$y = 2.39x - 1.39$
P. parasitica var. *nicotianae*	47 (41–52)	$y = 1.33x + 2.77$
mutant 83 (P1755)	665 (621–720)	$y = 3.73x - 5.55$
mutant 99 (P1756)	514 (484–544)	$y = 3.23x - 3.78$
mutant 120 (P1757)	535 (503–568)	$y = 3.35x - 4.17$
mutant 121 (P1758)	650 (569–767)	$y = 2.19x - 1.17$
P. parasitica var. *nicotianae*	<49	
mutant 1 (P1759)	270 (246–295)	$y = 2.51x - 1.12$
mutant 3 (P1761)	301 (274–331)	$y = 2.04x - 0.08$

[a] Mutant isolates were obtained by chemical mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine of encysted zoospores of the parental isolates listed above the mutant isolates.

[b] Values in parentheses indicate the 5% confidence interval. Regression data are derived from plotting percent inhibition on a probit scale versus log concentration. Correlation coefficient (r) ranged from 0.95 to 0.99 ($P = 0.05$).

[c] Mycelial growth of *P. parasitica* var. *nicotianae* (P1495) was inhibited 85% at the lowest concentration of HPO_3^{2-} used in the experiment (49 μg/ml).

TABLE 5. Concentration of phosphonate (HPO_3^{2-}) and incidence of disease in 1-mo-old tobacco seedlings following treatment with potassium phosphonate and inoculation with either HPO_3^{2-} -sensitive (P1352) or -resistant strains (P1755) of *Phytophthora parasitica* var. *nicotianae*

HPO_3^{2-} concentration of drench solution (μg/ml)[a]	HPO_3^{2-} concentration in tobacco stems (μg/g fresh weight)[b]	Disease rating[c]		Percent stem pieces infected[d]	
		P1352	P1755	P1352	P1755
0	0	2.6	3.0	100	100
195	215	0.6	3.0	60	100
390	279	0.6	2.9	4	70
780	484	0.0	2.5	0	67
1560	753	0.0	0.0	0	38

[a] Plants were treated by drenching with potassium phosphonate 36 hr before inoculation or extraction for analysis. Plants were grown in vermiculite in 355-ml styrofoam cups.

[b] Determined by high performance ion chromatography following water extraction of tobacco stems previously frozen with liquid nitrogen and ground to a powder. Phosphate content of the stem tissue was 3.2 mM.

[c] Disease rating is based on a scale where 0 = healthy and 3 = dead plants. Disease was evaluated 5 days after inoculation. Least significant difference ($P = 0.05$) is 0.9 for both P1352 and P1755. Data for disease rating and percent stem pieces infected are from two experiments.

[d] Percent stem pieces infected was calculated from the number of stem sections (0.4 cm in length) from which *P. parasitica* var. *nicotianae* was recovered following plating out of stem pieces on selective media. $P = 0.001$ with $X^2 = 77.5$ (P1352) and $P = 0.01$ with $X^2 = 18.7$ (P1755) for comparison of number of stem pieces infected at each concentration of HPO_3^{2-}.

The treatments ranged from 195 to 1,560 μg/ml of HPO_3^{2-}, whereas the corresponding concentrations measured by HPIC in the tobacco stems were 215–753 μg/g fr. wt. (Table 5). The P_i 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_3^{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_3^{2-}-sensitive isolates were similarly inhibited by HPO_3^{2-} in vitro and in vivo. On the other hand, the resistant isolates were only partially inhibited by high concentrations of HPO_3^{2-} in culture media or in plant tissue (Table 6).

Effect of AOA on disease control by HPO_3^{2-} in tobacco seedlings and on fungal uptake of HPO_3^{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_3^{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_3^{2-}, 63% of the stem pieces were infected. In tobacco seedlings treated with 390 μg/ml of HPO_3^{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_3^{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_3^{2-}, the HPO_3^{2-} concentration in the mycelium was 2.03 and 2.00 μg/g fr. wt. as determined by HPIC and radiolabel methods, respectively. In the presence of 0.5 mM AOA, uptake of HPO_3^{2-} was inhibited 77–80% after 4 hr as determined by both methods. There were no significant differences in HPO_3^{2-} quantification between the two methods.

Effects of phosphate level on the antifungal activity of HPO_3^{2-}. An HPO_3^{2-}-sensitive isolate (P1319) and -resistant mutant (P1361) of *P. capsici* and isolate P1352 of *P. parasitica* var. *nicotianae* were grown on RMSM with 5, 10, 15, or 45 mM potassium phosphonate (P_i) using a range of HPO_3^{2-} concentrations. With *P. capsici* (P1319), HPO_3^{2-} was more inhibitory to mycelial growth in the presence of 5 mM P_i than in the presence of 15 or 45 mM P_i (Fig. 2). The respective EC_{50} values at 5, 10, 15, and 45 mM P_i were 77, 135, 184, and 186 μg/ml of HPO_3^{2-}. The HPO_3^{2-}-resistant mutant of *P. capsici* (1361) also was more inhibited with 5 mM P_i than with 15 or 45 mM P_i (Fig. 3). The EC_{50} values at 5, 15, and 45 mM P_i were 344, 607, and 503 μg/ml of HPO_3^{2-}, respectively. Each EC_{50} value was statistically different from the other two values ($P = 0.05$).

Increasing the P_i concentration from 5 to 15 mM reduced the antifungal activity of HPO_3^{2-} toward *P. parasitica* var. *nicotianae* (P1352) (Fig. 4). The respective EC_{50} values with 5 and 15 mM P_i were 55 and 123 μg/ml of HPO_3^{2-}. However, further increasing the P_i 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_3^{2-} at 5 and 15 mM.

TABLE 6. Comparison of in vivo and in vitro antifungal activity of phosphonate (HPO_3^{2-}) toward growth of HPO_3^{2-}-sensitive and -resistant isolates of *Phytophthora capsici* and *P. parasitica* var. *nicotianae*

Isolate[a]	Growth medium[b]	HPO_3^{2-} concentration[c]	Percent inhibition[d]
P. capsici (P1319)	Tomato leaflet,	78–100[e]	61
	RMSM	77	50
P. capsici (P1361)	Tomato leaflet,	484–642[e]	37
	RMSM	344	50
P. parasitica var.	Tobacco stem,	279	96
nicotianae (P1352)	0.5% CMA	279	86
P. parasitica var.	Tobacco stem,	753	62
nicotianae (P1755)	0.5% CMA	665	50

[a] P1319 and P1352 are HPO_3^{2-}-sensitive parental isolates. P1361 and P1755 are HPO_3^{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_3^{2-} was determined by measuring radial growth on either Ribeiro's modified synthetic medium (RMSM) containing 5 mM P_i or on 0.5% cornmeal agar (CMA) at various concentrations of HPO_3^{2-}.

[c] Concentrations of HPO_3^{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_3^{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 phosphonate. Values for growth on culture media represent the level of inhibition of radial growth at the stated concentrations of HPO_3^{2-}.

[e] The first value was determined using HPIC and the second value was determined from scintillation counting of tritium-labeled HPO_3^{2-} in leaflet extracts.

DISCUSSION

Tomato leaflets containing 88 μg/ml of HPO_3^{2-} and tobacco stems containing 215 μg/ml of HPO_3^{2-} did not develop symptoms when inoculated with HPO_3^{2-}-sensitive isolates of *P. capsici* or *P. parasitica* var. *nicotianae*. On the other hand, HPO_3^{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_3^{2-}. The most obvious interpretation of these results is that HPO_3^{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_3^{2-} in tomato leaflets and tobacco seedlings treated with fosetyl-Al agrees with previous reports suggesting a rapid degradation of fosetyl-Al to HPO_3^{2-} in plant tissue (7,11,22,23,27). This strengthens the concept that

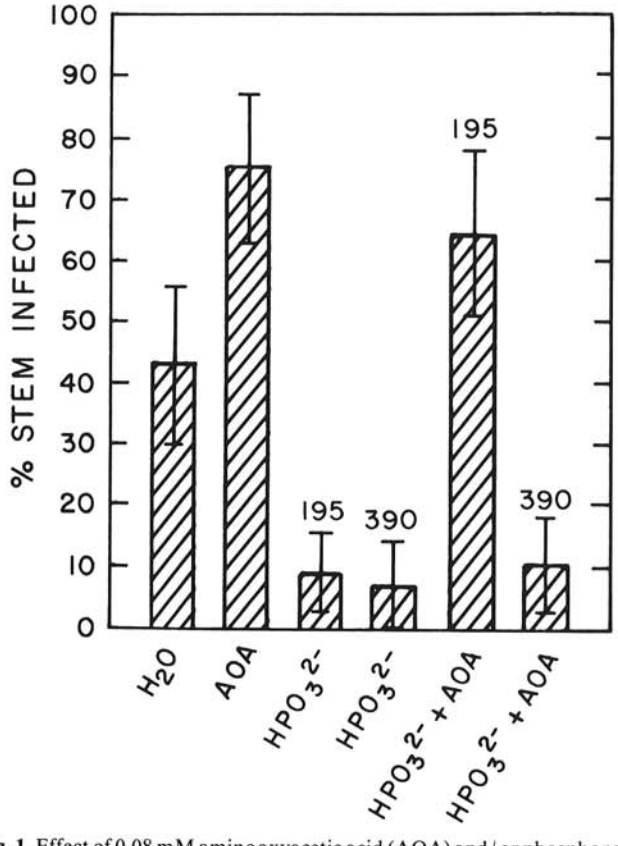

Fig. 1. Effect of 0.08 mM aminooxyacetic acid (AOA) and/or phosphonate on disease control in tobacco seedlings inoculated with zoospores of *Phytophthora parasitica* var. *nicotianae* isolate P1352. The AOA concentration in all treatments with AOA was 0.08 mM. The concentration of phosphonate (HPO_3^{2-}) used, expressed in micrograms per milliliter, is shown above the histogram bars. The 95% confidence intervals are shown for each treatment.

HPO_3^{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_3^{2-} measured in plant tissues and fungal mycelium by both HPIC and radiolabel 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 $[^3H]$-PO_3^{2-}, the stability of the tritium label had not been previously demonstrated. For this reason, buffered solutions (pH 6.2) of $[^3H]$-PO_3^{2-} and extracts of plants treated with $[^3H]$-PO_3^{2-} were fractionated by HPIC. The association of 93–95% of the radioactivity with the HPO_3^{2-} peak fraction even after 8 mo of storage as potassium phosphonate at pH 6.2 provides evidence that the tritium label is bound to the phosphorous atom in $[^3H]$-PO_3^{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_3^{2-} in vitro (28). Although the mode of action of HPO_3^{2-} is still unknown, the ability to obtain HPO_3^{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_3^{2-} and ethyl phosphonate was only partially reduced by AOA in tomato leaflets inoculated with *P. capsici*. Uptake of HPO_3^{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_3^{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_3^{2-} was ineffective in preventing stem infection by *P. parasitica* var. *nicotianae*, whereas 390 μg/ml of HPO_3^{2-} resulted in disease control. Thus, in tomato and tobacco, AOA only reversed the antifungal activity of HPO_3^{2-} when relatively low concentrations were applied. Lesion size on tomato 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_3^{2-} uptake by the pathogen can explain the need for higher levels of HPO_3^{2-} for disease control in AOA-treated plants.

In some cases a high level of P_i has been shown to reduce the in vitro antifungal activity of HPO_3^{2-} and ethyl phosphonate (5,16). Reduction in efficacy with high P_i was far greater with ethyl phosphonate than with HPO_3^{2-} (16). In the current study, the HPO_3^{2-}-sensitive and -resistant strains of *P. capsici* and a sensitive isolate of *P. parasitica* var. *nicotianae* were less inhibited in vitro by HPO_3^{2-} as the P_i level increased from 5 to 15 mM. However, at some concentrations of HPO_3^{2-}, inhibition of mycelial growth in vitro was greater at 45 mM P_i 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 phosphonate was enhanced when higher concentrations of P_i were included in the treatments. Bompeix and Saindrenan (5) found that *Phytophthora* species varied in the

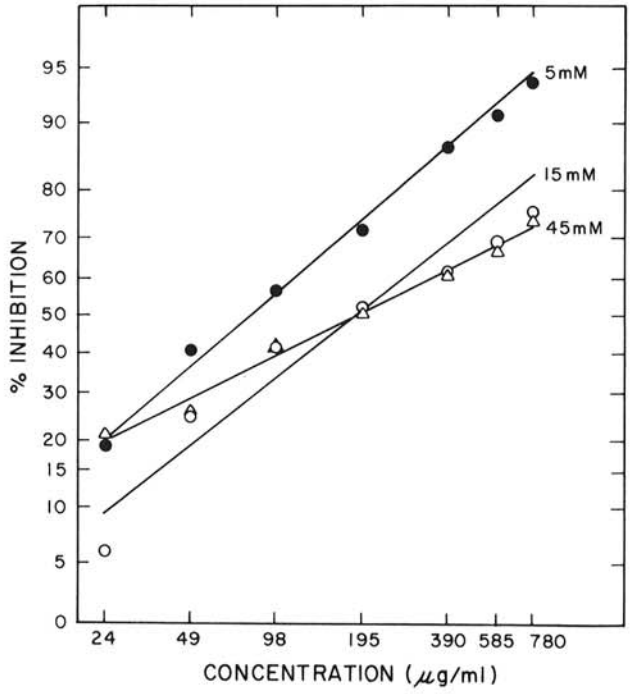

Fig 2. Dosage response curve for *Phytophthora capsici*, isolate P1319, on Ribeiro's synthetic medium containing different concentrations of phosphonate (μg/ml) and either 5, 15, or 45 mM potassium phosphate. Correlation coefficients (r) ranged from 0.96 to 0.99 and all were significantly positive ($P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

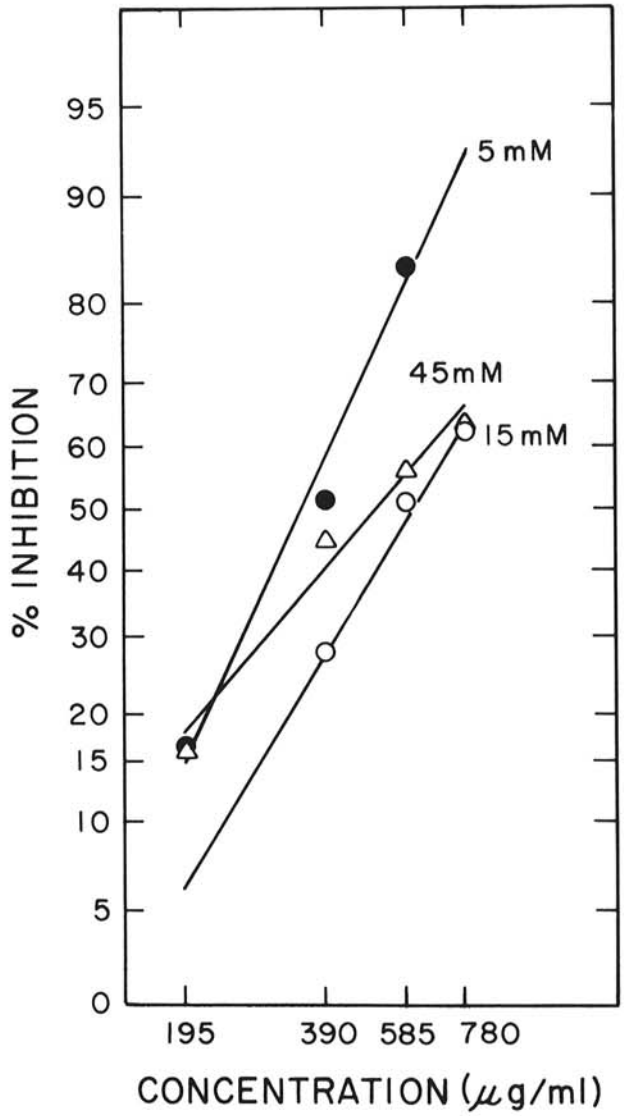

Fig. 3. Dosage response curve for a phosphonate (HPO_3^{2-})-resistant mutant (P1361) of *Phytophthora capsici* (P1319) on Ribeiro's synthetic medium containing different concentrations of HPO_3^{2-} (μg/ml) and either 5, 15, or 45 mM potassium phosphate. Correlation coefficients (r) ranged from 0.95 to 0.97 and all were significantly positive ($P = 0.05$).

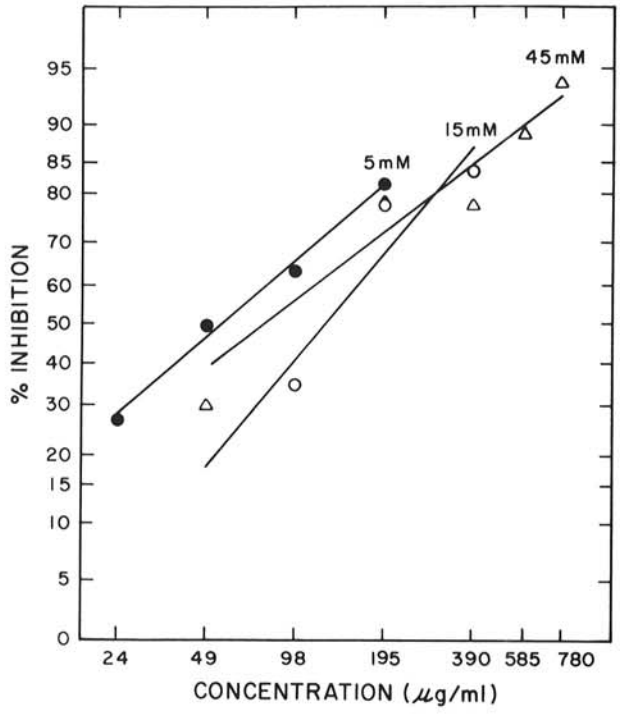

Fig. 4. Dosage response curve for *Phytophthora parasitica* var. *nicotianae* isolate P1352 on Ribeiro's synthetic medium containing different concentrations of phosphonate (μg/ml) and either 5, 15, or 45 mM potassium phosphate. Correlation coefficients (r) ranged from 0.91 to 0.98 and all were significantly positive ($P = 0.05$).

degree to which P_i reduced the antifungal activity of HPO_3^{2-} toward them. They reported that P_i had little or no impact on the efficacy of HPO_3^{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_i concentration on the efficacy of phosphonate fungicides probably have been overemphasized. For instance, P_i concentrations in plant tissues used in this study ranged from 2 to 9 mM. The concentrations of HPO_3^{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_3^{2-} in tomato and tobacco tissues treated with fosetyl-Al or potassium phosphonate suggests that HPO_3^{2-} is the active principle in preventing infection with *P. capsici* and *P. parasitica* var. *nicotianae*. The inefficacy of fosetyl-Al and HPO_3^{2-} for disease control in plants inoculated with HPO_3^{2-}-resistant mutants provides strong evidence for a direct antifungal mode of action for phosphonate fungicides.

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