The effect of phosphonate on the acid-soluble phosphorus components in the genus *Phytophthora*

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The high concentrations of short-chain soluble polyphosphates, previously reported in *Phytophthora palmivora*, have been found in six other species of *Phytophthora*, and appear to be characteristic of this genus. The distribution of phosphorus among the various acid-soluble pools was similar in all species except *P. infestans*, which accumulated less polyphosphate than other species when grown in high phosphate medium, and contained several unique and as yet unidentified organic phosphorus compounds. When grown in the presence of potassium phosphonate sufficient to reduce growth by 50%, all species showed an increased accumulation of phosphorus in both pyrophosphate and polyphosphate, without parallel increases in sugar phosphate or nucleotide phosphorus pools. The internal concentration of phosphonate required to produce 50% inhibition varied with species, ranging from 4.6 to 52 $\mu\text{mol (g dry wt)}^{-1}$. Assimilation of orthophosphate was only slightly reduced in the presence of phosphonate, resulting in an increased concentration of phosphorus per unit mass. Metalaxyl, which also inhibits growth of *Phytophthora* spp. did not cause accumulation of either pyro- or polyphosphate. Phosphonate treatment also resulted in the formation of a compound identified as isohypophosphate, a metabolite of phosphonate not previously reported in *Phytophthora*. Taken together, these observations suggest that the primary site of phosphonate inhibition in *Phytophthora* spp. lies in the metabolism of pyrophosphate.

Keywords: *Phytophthora* spp., phosphorus assimilation, phosphonate, ³¹P NMR

INTRODUCTION

Phosphonates, applied as either the potassium salts of phosphonic acid (Folia-R-Fos) or aluminium salts of alkyl phosphonates (Fosetyl Al, Aliette), control several diseases caused by species of pseudofungi belonging to the class Oomycetes. However, there is evidence that the spectrum of activity of phosphonate is broader and includes species from classes of organism other than Oomycetes (Chase, 1993; Davis *et al.*, 1993; Heaton & Dullahide, 1990). While there is strong evidence that the primary site of phosphonate action lies within the pathogen and not the host (Fenn & Coffey, 1984), this site has not been identified.

Abbreviations: polyP, polyphosphate; pyroP, pyrophosphate; LPR, low-phosphate Ribeiro medium; NDPG, nucleoside diphosphate glucose; PCA, perchloric acid; IHP, isohypophosphate.

Relatively high concentrations (0.1–3 mM) of phosphonates are required to reduce growth of target organisms in culture, although the anion appears to be effective in the plant at lower concentrations (Smillie *et al.*, 1989). There is indirect evidence suggesting that phosphonates alter the wall structure of *Phytophthora* spp. (Dercks & Buchenauer, 1987; Dunstan *et al.*, 1990; Guest, 1986). Such changes could affect the interaction between host and parasite, enhancing the activity of the host plant's dynamic defence response as observed in the presence of phosphonates (Afek & Szejnberg, 1989; Bompeix *et al.*, 1980; Nemostothy & Guest, 1990). However, it is not known whether the primary site of phosphonate action is restricted to the metabolic pathway leading to cell wall formation, or whether the effect on wall formation is secondary, resulting from a phosphonate-induced alteration in metabolism at another site.

Fig. 1. ^{31}P NMR spectra at 81 MHz of PCA extracts of *P. citricola* (a) grown in LPR and (b) grown in LPR in the presence of 0.3 mM phosphonate. Peak assignments: 1, methylene diphosphonic acid (external standard); 2, phosphonate; 3, phosphomonoesters, e.g. fructose 6-phosphate, glycerophosphate etc, individual resonances were not assigned; 4, inorganic phosphate; 5, terminal P of polyphosphates with $> 4\text{P}$; 6, terminal P of tetrapolyP and $\gamma\text{-P}$ of nucleoside triphosphates such as ATP; 7, terminal P of tripolyP; 8, pyroP; 9, $\alpha\text{-P}$ of ATP and other nucleoside tri- and diphosphates, P of UDPG, and perhaps P of NAD; 10, P of UDPG; 11, nonterminal P of inorganic polyP and $\beta\text{-P}$ of nucleoside triphosphates.

Alterations in levels of nucleotides and pyrophosphate (Griffith *et al.*, 1990) were observed in mycelium of *P. palmivora* P113 grown on phosphonate and changes in nucleotide levels were also observed in *P. citrophthora* (Barchietto *et al.*, 1992) indicating that phosphonate causes significant perturbations in phosphorus metabolism. In this paper, the effects of phosphonate on the levels of acid-soluble phosphorylated metabolites in a number of species of *Phytophthora* are reported.

METHODS

Reproducibility. All data presented in this paper are representative and are based on at least three experiments except where indicated. Where data points are reported as being significantly different, the test was made by Student's *t*-test.

Strains. The following cultures originated from the *Phytophthora* culture collection at the University of California, Riverside, and were obtained from Professor Michael Coffey: *Phytophthora palmivora* P113 (deposited as ATCC 26286, Tokunaga & Bartnicki-Garcia, 1971), an isolate resistant to phosphonate; *P. palmivora* P376, an isolate particularly sensitive to phosphonate (Dolan & Coffey, 1988); *P. citricola*, P1946; *P. melonis*, P3239; and *P. mirabilis*, P3010. *Phytophthora nicotianae* M3049 was isolated from tobacco (Smillie *et al.*, 1989), while *P. infestans* 88198 is a metalaxyl-sensitive potato isolate obtained from the Netherlands (Grant *et al.*, 1992).

Media and growth conditions. All cultures except *P. infestans* and *P. mirabilis* were maintained on V-8 agar and transferred to low phosphate Ribeiro agar medium (Griffith *et al.*, 1989) prior to use as inocula in these experiments. *Phytophthora infestans* and *P. mirabilis* were maintained on rye seed infusion agar and grown in rye seed infusion broth (Caten & Jinks, 1968). Other species were grown in liquid Ribeiro medium (LPR) containing 0.1 mM phosphate, buffered with 50 mM MES buffer adjusted to pH 6.2 with KOH (Griffith *et al.*, 1989). Thiamin and, where indicated, potassium phosphonate or metalaxyl were added aseptically after autoclaving.

Cultures were routinely grown in darkness at 26 °C for 6 or 7 d, which corresponded to the transition from exponential to stationary growth phase. At that time phosphate was almost exhausted in the LPR growth medium. Other growth periods were used where indicated. *P. infestans* was normally grown at 20 °C, its optimum temperature.

The level of phosphonate used was chosen to yield approximately 50% inhibition at the phosphate level supplied. In practice the inhibition obtained varied from 51–65%. The specific level of phosphonate applied to each species except *P. infestans* is specified in Table 1. The EC_{50} level for *P. infestans* 88198 grown on rye seed infusion broth was 2 mM.

Harvesting and extraction procedures. Harvesting and extraction of mycelia were carried out as described by Niere *et al.* (1990) with the exception that the freeze-thaw step was omitted. This change did not affect the ^{31}P NMR spectra of the extracts obtained.

NMR preparation, conditions and calculations. Freeze-dried PCA extract was dissolved in $^2\text{H}_2\text{O}$ (0.5 ml), 0.1 M EDTA (0.5 ml, pH 7.5) and water (0.5 ml). The resulting solution was placed in a 10 mm o.d. NMR tube and the pH adjusted to 7.8 with 0.1 M KOH. The final volume of the sample was adjusted to 2.20 ± 0.05 ml. All ^{31}P NMR experiments were conducted using a Bruker AC200-F instrument (operating frequency 81 MHz) at a temperature of 295 ± 1 K. Spectra were normally accumulated using 45° pulses and 7 s cycle times in order to ensure total relaxation of all ^{31}P nuclei. The spectral width of 6000 Hz was represented by 8 K data points. Spectra were normally run fully coupled.

Chemical shifts were referenced to a methylene diphosphonic acid (0.1 M in a 1 mm concentric capillary tube) signal set at 16.85 p.p.m. downfield from 85% (v/v) H_3PO_4 . The same reference sample was used as a standard to calculate absolute concentrations of different types of phosphorus in the NMR sample. These concentrations, the NMR sample volume and

Table 1. Acid-soluble phosphorus pools in mycelium of *Phytophthora* spp. in the absence or presence of sub-toxic levels of phosphonate

Results are expressed as (a) μmol phosphorus (g mycelial dry wt) $^{-1}$ and (b) % of total acid-soluble phosphorus. Standard deviations are shown in parentheses.

Species	Days growth	Mean polyP chain length	PolyP		PyroP		P_i		Nucleotide P		Sugar phosphate		Total P	Phosphonate	External phosphonate (mM)
			a	b	a	b	a	b	a	b	a	b	a	a	
<i>P. citricola</i> (control)	6	5.0 (0.3)	41.2 (7.7)	54 (7)	2.6 (0.2)	3.5 (0.6)	9.3 (1.1)	12 (1)	9.3 (1.1)	12 (2)	8.5 (1.6)	11 (2)	76.9 (10.4)		
<i>P. citricola</i> (treated)	6	4.0 (0.3)	149.6 (2.0)	67 (1)	24.2 (7.7)	11 (3)	23.5 (1.0)	9 (1)	10.9 (3.5)	5 (2)	13.5 (4.0)	6 (2)	223.7 (0.5)	23.5 (1.5)	0.3
<i>P. melonis</i> (control)	7	5.2 (0.3)	24.0 (8.3)	44 (9)	1.9 (0.7)	3.8 (2.0)	9.0 (2.8)	18 (8)	6.8 (1.7)	13 (1)	7.6 (0.7)	15 (1)	52.7 (8.3)		
<i>P. melonis</i> (treated)	7	4.3 (0.1)	85.3 (14.9)	63 (4)	11.8 (0.8)	9 (2)	14.4 (3.4)	11 (4)	7.7 (1.4)	6 (0.5)	11.1 (1.9)	8 (1)	134.6 (15.5)	4.6 (1.3)	0.4
<i>P. nicotianae</i> (control)	7	6.9 (0.6)	44.4 (7.0)	58 (6)	1.6 (0.1)	1.9 (0.3)	10.8 (5.0)	11 (4)	5.5 (1.4)	10 (3)	8.6 (0.9)	13 (2)	73.4 (2.7)		
<i>P. nicotianae</i> (treated)	7	4.4 (0.1)	134.0 (9.6)	71 (4)	15.4 (4.0)	8 (2)	14.2 (3.4)	8 (2)	7.1 (0.8)	4 (0.5)	14.6 (0.4)	8 (1)	188.5 (4.5)	21.8 (9.0)	0.6
<i>P. palmivora</i>															
P113 (control)	7	5.5 (0.2)	24.9 (8.2)	52 (4)	1.4 (0.7)	3.3 (0.6)	3.9 (0.8)	10 (3)	5.6 (0.7)	12 (2)	6.3 (1.9)	13 (1)	46.0 (13.0)		
P113 (treated)	7	4.0 (0.2)	102.9 (24.5)	60 (3)	27.2 (9.4)	15 (1)	19.6 (5.0)	11 (2)	6.5 (1.0)	4 (0.5)	12.0 (2.9)	7 (1)	174.1 (47.9)	38.8 (11.6)	1
P376 (control)	7	6.2 (0.5)	39.6 (9.3)	63 (6)	1.4 (0.6)	2.0 (0.5)	7.2 (3.8)	11 (5)	6.5 (0.8)	10 (1)	6.9 (0.6)	11 (2)	64.9 (12.5)		
P376 (treated)	7	3.6 (0.1)	101.3 (6.3)	49 (6)	40.9 (6.6)	20 (3)	35.4 (12.6)	18 (3)	6.7 (0.8)	3 (0.5)	16.9 (1.3)	8 (0)	206.0 (10.8)	51.4 (10.8)	0.25

the dry mass of the mycelium were then used to calculate the number of moles of different types of phosphorus per gram of mycelium. Peaks were assigned by reference to published chemical shifts (Navon *et al.*, 1979). In cases where chemical shift was dependent upon pH and metal ion concentration, assignment was checked by spiking the extract with authentic samples and varying the pH.

Calculations of the mean polyP chain length are as described by Niere *et al.* (1990) except that the contribution from pyroP was excluded in the current calculations.

Dry weights of mycelium extracted for use in the NMR studies varied. The spectra in Figs 1, 2, 3 and 5 have been scaled so that they represent extracts from equivalent mycelial masses.

Measurement of phosphate and glucose assimilation. Phosphate assimilation was determined by growing the organisms in ^{32}P -labelled phosphate (specific activity $1.5 \text{ MBq } \mu\text{mol}^{-1}$) and measuring the radioactivity present in aliquots of media at daily intervals, using Cerenkov counting. At completion of the assimilation experiments (normally 5–7 d), mycelia were harvested by filtration on to Whatman GF/C filters, washed, freeze-dried and weighed. The protocol for the measurement of glucose utilization was identical, except that D-[U- ^{14}C]glucose ($2 \text{ MBq } \mu\text{mol}^{-1}$) was supplied to the medium and radioactivity determined by liquid scintillation counting.

Measurement of respiration. Mycelium of *P. palmivora* was grown for 5 d in LPR at 26°C , separated from the growth medium on coarse nylon mesh and washed with fresh, sterile LPR. The washed mycelium was then blotted dry on filter paper and transferred to two conical flasks each containing 50 ml fresh LPR containing 5 mM D-[U- ^{14}C]glucose ($200 \text{ MBq } \mu\text{mol}^{-1}$) and fitted so that 200 ml min^{-1} of filtered, water-saturated air passed through each. Each flask was placed in a water bath at 26°C . The air stream was aspirated through 1 M sulfuric acid and collected in scintillation vials containing 3 ml of Soluene to absorb CO_2 . Vials were changed every 20 min. Scintillation fluid was then added and the radioactivity in each determined by scintillation counting in an LKB Rackbeta 1214, with an appropriate quench correction program. After 2.5 h, potassium phosphonate was added to one flask to bring the final concentration to 1 mM and water added to the second flask, and

the collection resumed. Experiments were continued for 15 h, and 15–25% of the added glucose was assimilated during this period. Since replication of treatments was not possible in these experiments, each experiment was repeated four times, using separate batches of mycelium.

RESULTS

NMR analysis of acid-soluble phosphorus pools in *Phytophthora* species

The spectra of the acid-soluble phosphorus components in control and phosphonate-treated *P. citricola* are shown in Fig. 1. Similar patterns of peaks were observed in the spectra of all other species of *Phytophthora* examined which included representatives from the different groups within the genus based on morphological characteristics (Waterhouse *et al.*, 1983). The quantitative distribution of phosphorus among the major acid-soluble pools in various species and isolates is shown in Table 1. A characteristic feature of all species was the presence of large amounts of low ($\text{DP} < 10$) molecular mass polyP, and the absence of high molecular mass polyP, a feature which appears to distinguish the genus. Analysis of single extracts from *P. cinnamomi* showed a similar pattern (data not shown).

Two closely related species, *P. infestans* and *P. mirabilis*, did not grow well on LPR. They grew better, but still poorly, in a defined medium containing 3 mM P_i , sterol and lecithin (Hohl, 1975) and in this medium had lower polyP pool sizes than observed in other species grown on LPR (0.1 mM P_i). The polyP pool in *P. infestans* was smaller than in any other species examined. In the sugar phosphate region, a new peak appeared at 5.5 p.p.m. and the relative intensity of the peak at 5.0 p.p.m. was greater than that previously observed in other species. Neither of these peaks was assigned, the chemical shift of the latter being close to but not identical with those of glucose 6-phosphate (Fig. 2).

Fig. 2. ^{31}P NMR spectrum of PCA extract of *P. infestans* 88198 grown in enriched Ribeiro medium (P_i concn 3 mM). Peak assignments are as in Fig. 1.

More vigorous growth of *P. infestans* and *P. mirabilis* on rye broth (0.25 mM P_i) gave mycelia with a phosphorus distribution similar to that of other species grown on LPR (0.1 mM P_i) (Fig. 3a) but without the chain-lengthening or increased accumulation of polyP observed when other species were grown in rye (Fig. 3b) or other media with phosphate levels greater than 0.1 mM (Niere *et al.*, 1990).

Effect of phosphonate on the distribution of acid-soluble phosphorus

In all species and isolates examined, phosphonate-induced growth inhibition resulted in several striking changes in acid-soluble phosphorus pools (Tables 1 and 2, Fig. 1b). There was both a very large increase in the amount of acid-soluble phosphorus per unit mass of mycelium and a change in the distribution of phosphorus between the various pools. PolyP increased three- to fivefold, with concentrations reaching 85–150 μmol (g dry wt mycelium) $^{-1}$. The increases in the pyroP pools were even more striking, showing 7–35-fold increases [12–41 μmol

Fig. 3. ^{31}P NMR spectra of PCA extracts of (a) *P. infestans* 88198, (b) *P. palmivora* P113 grown in rye seed infusion broth. Peak assignments are as in Fig. 1.

(g dry wt mycelium) $^{-1}$. There were smaller increases in the sugar phosphate pools (up to twofold), but only little or no increase in nucleotide phosphorus. The

Table 2. Percentage changes in phosphorus pools of *Phytophthora* spp. treated with phosphonate

Standard deviations are shown in parentheses.

Species	Days growth	Mean polyP chain length	PolyP	PyroP	P_i	Nucleotide P	Sugar phosphate	Total P	Growth inhibition (%)
<i>P. citricola</i>	6	−12 (1)	252 (9)	785 (200)	113 (47)	14 (3)	64 (9)	173 (26)	53 (12)
<i>P. melonis</i>	7	−18 (3)	293 (60)	600 (180)	73 (56)	15 (9)	46 (12)	157 (12)	65 (10)
<i>P. nicotianae</i>	7	−40 (5)	231 (40)	851 (200)	49 (38)	35 (19)	70 (12)	157 (4)	52 (3)
<i>P. palmivora</i>									
P113	7	−27 (5)	415 (60)	2008 (534)	403 (157)	16 (5)	98 (37)	281 (34)	51 (6)
P376	7	−42 (4)	258 (50)	3417 (1363)	639 (511)	5 (11)	146 (25)	224 (37)	58 (6)

Table 3. Amount of phosphorus in different acid-soluble pools in *P. palmivora* P113

P. palmivora P113 was grown for 3, 5, 7 and 10 d in LPR alone or in LPR containing 1 mM phosphonate. The results are expressed as $\mu\text{mol P (g mycelial dry wt)}^{-1}$.

Days growth	1 mM phosphonate	Mean polyP chain length	PolyP	PyroP	P _i	Nucleotide P	Sugar phosphate	Total acid-soluble P	Phosphonate	Growth inhibition (%)
3	—	4.8	143.5	5.7	16.3	18.2	16.3	205.7	—	47
	+	3.7	135.1	17.6	23.2	12.1	21.3	213.0	10.4	
5	—	5.5	46.7	1.6	5.3	8.5	7.8	73.0	—	46
	+	4.3	126.9	16.2	17.6	11.4	15.3	190.0	31.2	
7	—	5.2	24.2	1.0	4.3	5.7	5.4	42.2	—	53
	+	4.3	92.3	18.7	12.8	7.0	8.8	143.0	30.2	
10	—	4.9	19.0	1.0	2.4	4.0	3.3	31.0	—	64
	+	3.8	101.6	30.2	17.7	6.1	12.7	175.0	38.8	

orthophosphate pool also increased with the *P. palmivora* isolates (P113 and P376) showing the largest changes.

Fully coupled ^{31}P NMR spectra of PCA extracts of phosphonate-treated mycelia (Fig. 1b) contained a small doublet (J 17 Hz) at -7.8 p.p.m. which does not appear to have been reported previously in any ^{31}P NMR spectra of biological extracts. In proton-decoupled spectra this doublet was replaced by a more intense doublet (J 17 Hz) at -3.8 p.p.m. This suggested that the doublet arose from a ^{31}P nucleus coupled to both an H (J approx. 640 Hz) and to another P (J 17 Hz). The simplest molecule incorporating this arrangement is isohypophosphate (IHP):

The observed chemical shifts and coupling constants are consistent with those previously reported for IHP (Van Wazer, 1958). The fully coupled spectrum of the IHP anion consists of a doublet of doublets (phosphonate P, centred at -1 p.p.m.) and a doublet (phosphate P, centred at -3 p.p.m.). In the fully coupled spectrum of the mycelial extract, the phosphate signal of IHP would be obscured by the low-field terminal polyP peaks, while the doublet comprising the low-field half of the IHP phosphonate signal would be obscured by the high-field half of the very large phosphonate doublet at 0.0 p.p.m. To confirm the identity of the phosphorylated metabolite, X, a mixture of IHP, pyroP, phosphonate and phosphate was prepared as described by Blaser & Worms (1959). The mycelial acid-extract was fractionated through a Dowex 1 $\times$ 8-formate anion exchange column and a fraction consisting of X, pyroP, phosphonate and phosphate (as indicated by ^{31}P NMR) was isolated, neutralized and freeze-dried. Proton and ^{13}C NMR spectra of this fraction indicated that the only organic material present

was residual formic acid. ^{31}P NMR spiking experiments of this fraction with the synthetic mixture containing IHP confirmed that X was isohypophosphate. Isohypophosphate was never observed in mycelial extracts which had not been exposed to phosphonate. There were no IHP peaks in the NMR spectrum of the original phosphonate stock.

In those cases where internal levels of phosphonate were very low (*P. melonis* and *P. infestans*), no IHP peaks were detectable. In order to determine whether this compound was simply an artifact formed by reaction of phosphonate with phosphate or pyrophosphate during the extraction procedure, mixtures of phosphonate with the growth medium and phosphonate with pyrophosphate were subjected to the same freeze-drying and extraction procedures as the phosphonate-treated mycelia. No isohypophosphate peaks could be found in the ^{31}P NMR spectra of the resulting mixtures.

Variation in size of internal phosphonate pools

Internal phosphonate pool sizes ranged from 4.6 – 51.4 $\mu\text{mol (g dry wt)}^{-1}$. There was no consistent relationship between the internal pool sizes and the concentration of phosphonate supplied. For example, the phosphonate pool sizes in *P. palmivora* P113 and P376 were similar, despite a fourfold difference in external phosphonate concentrations. Phosphonate pool sizes in *P. nicotianae* and *P. citricola* were approximately half those observed in the *P. palmivora* isolates, and the pool size in *P. melonis* was very much smaller than in any other isolates examined. Although this may be due in part to *P. melonis* containing less soluble material (g dry wt^{-1}) than the other species, the amount of phosphonate measured as a fraction of total acid-soluble phosphorus was still only half that observed for *P. nicotianae* and *P. citricola*. These observations support the conclusions of Griffith *et al.* (1993) who not only found differences between isolates in their capacity to exclude phosphonate at the level of the

Fig. 4. Phosphate uptake in the absence (open symbols) or presence (filled symbols) of phosphonate [1 mM (a), 0.3 mM (b, c)]: (a) *P. palmivora* P113; (b) *P. palmivora* P376; (c) *P. citricola*.

phosphate–phosphonate translocator, but also differences between isolates in their sensitivity to internal phosphonate pools of similar magnitude.

When *P. infestans* was grown on rye broth in the presence of phosphonate, the amount of phosphonate accumulated [$14.4 \mu\text{mol (g dry wt)}^{-1}$] was smaller than in other species inhibited to a comparable extent although similar increases in pyroP and polyP were observed.

Effect of culture age on phosphorus distribution

A single set of analyses made on *P. palmivora* P113 harvested after 3, 5, 7 and 10 d growth in LPR showed the effect of culture age on the distribution of phosphorus between the various pools (Table 3). Under the conditions of the experiment, 60 % of external P_i was assimilated by day 3 and more than 90 % by day 5 (see Fig. 4a), although the mycelial mass continued to increase until day 10. As a consequence, there was a significant fall in the total acid-soluble phosphorus expressed on a dry weight basis as the culture aged, with the maximum change occurring between days 3 and 5. There was only a small shift in distribution of phosphorus from acid-soluble inorganic to organic pools over this period, with 81 % of acid-soluble phosphorus in the inorganic pool at day 3, 73 % at day 5 and 71 % at day 10. These results are not consistent with the hypothesis that polyP functions as a phosphorus reserve in this genus. In a separate experiment, mycelium grown for 10 d under these conditions, washed and then transferred to new medium from which P_i was omitted, failed to increase in mass. This suggests that the accumulation of polyP is not a function of culture age, and that once accumulated, polyP cannot be utilized as an internal source of phosphorus when external sources are depleted.

The presence of phosphonate had two effects. The first was that the total pool size of acid-soluble phosphorus

Table 4. Effect of phosphonate on phosphate assimilation by *Phytophthora* spp.

Species	Phosphonate (mM)	Phosphate assimilated ($\mu\text{mol g}^{-1}$)
<i>P. palmivora</i> P113	0	196
	1.0	285
<i>P. palmivora</i> P376	0	328
	0.3	544
<i>P. palmivora</i> P7228	0	351
	0.3	453
	1.0	363
<i>P. citricola</i>	0	200
	0.3	428

was much higher at all times and that this was particularly apparent after day 3 (Table 3). The second was that the relative increase in the pyroP pool was observed even at day 3, well before growth became P_i limited, and before the relative increases in polyP became apparent.

Phosphate assimilation

The accumulation of more acid-soluble phosphate (g dry wt^{-1}) in the presence of phosphonate than in untreated mycelia (Table 4) was shown to result from a differential effect of phosphonate on the rate of P_i assimilation and growth. Phosphonate did reduce the rate of P_i assimilation, and the limiting concentration, $4 \mu\text{M}$, was reached 24 h later than in control cultures (Fig. 4). However, the reduction in growth in the phosphonate-treated cultures was much greater than the reduction in P_i assimilation, resulting in higher total phosphorus (g dry wt^{-1}).

Table 5. Effect on metalaxyl on phosphate uptake by *Phytophthora palmivora* P113

	Mycelial mass (mg)	Phosphate (μmol)	Phosphate assimilated (μmol g ⁻¹)
Control	22.3 ± 1.6	4.7 ± 0.1	200
Metalaxyl 0.05 mg l ⁻¹	12.1 ± 1.7	3.5 ± 0.3	300
Metalaxyl 0.1 mg l ⁻¹	4.0 ± 1.8	1.5 ± 0.4	300

Effect of metalaxyl

To test whether these effects of phosphonate were specific, or the result of general inhibition of growth, the effect of sub-toxic concentrations of metalaxyl, an acylalanine fungicide (Schwinn & Staub, 1987) was examined using *P. palmivora* P113. Metalaxyl is considered to act at the level of RNA polymerase (Davidse & Van denBerg Velthuis, 1989) and isolates of *Phytophthora* resistant to metalaxyl do not show cross-resistance to phosphonate (Bower & Coffey, 1985), suggesting that the two fungicides do not share a common site of action.

When *P. palmivora* P113 was grown in the presence of metalaxyl (0.05 and 0.1 μg ml⁻¹), growth was inhibited 50% and 80% respectively (Table 5). The effect on P_i assimilation was comparable to that observed when phosphonate inhibited growth, suggesting that this was not a specific effect. However, PCA extracts of mycelium

grown in the presence of metalaxyl showed none of the changes in pyroP and polyP characteristic of phosphonate-treated *P. palmivora* P113 (Fig. 5) which suggests that these are specific effects.

Effect of phosphonate on glucose assimilation

The conversion of phosphate to pyroP and polyP requires the expenditure of energy to form each phosphoanhydride bond. The requirement must be met either by an increase in the rate of glucose oxidation or a diversion of energy from other synthetic pathways. Growth of *P. palmivora* P113 and P376, and *P. citricola* in the presence of radio-labelled glucose showed no increase in glucose assimilation during growth in the presence of phosphonate (Table 6). Glucose assimilation (g dry wt)⁻¹ was reduced in proportion to the reduction in mycelial mass, except in the case of *P. citricola* where it was reduced proportionately more.

When phosphonate (1 mM) was supplied to actively growing cultures of *P. palmivora* P376, washed and resuspended in fresh LPR an inhibition of CO₂ evolution was observed within 1 h of addition of phosphonate (Fig. 6). Addition of phosphonate to *P. palmivora* P113 under identical conditions resulted in either no effect or very slight inhibition of respiration (data not shown). This difference would be predicted, since assimilation of phosphonate is more rapid into P376 than it is into P113 under the experimental conditions used (Griffith *et al.*, 1993). We have never observed stimulation of respiration in the presence of phosphonate.

Fig. 5. ³¹P NMR spectrum of PCA extract of *P. palmivora* P113 grown (a) in LPR; (b) in LPR plus 0.05 μg l⁻¹ metalaxyl.

Table 6. Effect of phosphonate on consumption of glucose by *Phytophthora* spp.Results are expressed as means $\pm$ SD.

Species	Phosphonate (mM)	Mean glucose used per flask (μ mol)	Mean dry weight per flask (mg)	Glucose use on a dry wt basis (mmol g^{-1})
<i>P. palmivora</i> P113	0	2.64 $\pm$ 0.13	22.76 $\pm$ 0.78	0.12 $\pm$ 0.01
	1.0	1.30 ^a $\pm$ 0.10	12.47 ^a $\pm$ 0.37	0.10 $\pm$ 0.01
<i>P. palmivora</i> P376	0	2.23 $\pm$ 0.16	18.59 $\pm$ 1.20	0.12 $\pm$ 0.01
	0.3	1.02 ^a $\pm$ 0.14	9.60 ^a $\pm$ 1.30	0.11 $\pm$ 0.01
<i>P. citricola</i>	0	2.21 $\pm$ 0.20	18.90 $\pm$ 0.73	0.12 $\pm$ 0.01
	0.3	0.44 ^a $\pm$ 0.12	5.00 ^a $\pm$ 0.44	0.07 ^b $\pm$ 0.04

^a Significantly different from control, $P < 0.001$; ^b significantly different from control, $P < 0.01$. **Fig. 6.** Effect of phosphonate on CO_2 evolution from actively growing *P. palmivora* P376. Phosphonate was added at the time indicated by the arrow. ○, Culture with no added phosphonate; ●, culture to which 1 mM phosphonate was added.

DISCUSSION

Analysis of acid-soluble phosphorus compounds by ^{31}P NMR showed a high degree of similarity in the type and concentration of compounds present in all species of *Phytophthora* examined, regardless of the group to which they are placed in the classification of Waterhouse *et al.* (1983). The exception to this observation was in the closely related (Galindo & Hohl, 1985) *P. infestans* and *P. mirabilis* where there were differences in polyP accumulation and where other, as yet unidentified, phosphorus compounds not found in the other species were observed. It is clear from our data that the presence of short-chain polyP is a characteristic feature of the genus, and not in any way unique to *P. palmivora*.

When growth of *Phytophthora* spp. was inhibited by phosphonate, the amount of phosphate assimilated per unit mycelial mass increased. This was not a specific effect,

since the same result was observed with metalaxyl inhibition. However, in the presence of phosphonate, most of the phosphate assimilated was converted to pyroP and polyP, while in the presence of metalaxyl it remained as P_i . The accumulation of pyroP and polyP therefore appears to be induced by phosphonate. This effect was evident in all species treated, regardless of their sensitivity to phosphonate.

The level of internal phosphonate which resulted in 50% inhibition of growth varied widely between the different species. The internal phosphonate pool sizes in *P. nicotianae* and *P. citricola* were only half those in *P. palmivora* and that in *P. melonis* was only 10%, although the extent of growth inhibition was essentially the same. The internal concentration of phosphonate required to inhibit growth of *P. infestans* was also low [$14.4 \mu\text{mol (g dry wt)}^{-1}$] although a strict comparison with other species is not possible because of the different growth media used. There are clearly differences in sensitivity between the different species at the internal site(s) at which phosphonate acts. These are in addition to differences in capacity to exclude phosphonate when phosphate is also present (Griffith *et al.*, 1993). Together these would determine the sensitivity to phosphonate at the whole organism level.

The increases in both pyroP and polyP induced by phosphonate must result from either an increased rate of synthesis or a decreased rate of utilization of these compounds. We know that the changes are rapid and that the increase in the pyroP pool size is one of the earliest effects observed in phosphonate-treated mycelium in *P. palmivora* P376 (J. M. Griffith, unpublished). Changes in pool size of nucleotide phosphorus are also observed in these conditions and in earlier experiments carried out over a short period under somewhat different conditions (Griffith *et al.*, 1990).

If pyroP breakdown is inhibited in the presence of phosphonate, a likely target site is the inorganic pyrophosphatase, which is abundant in the genus (Sysuev *et al.*, 1978) or a V-type proton translocating pyrophosphatase such as is found in the vacuolar membrane of

plants (Rea & Poole, 1993). To account for the parallel increase in accumulation of polyphosphate, one needs to postulate phosphonate inhibition of the polyphosphate phosphatase which hydrolyses low molecular mass polyP, and is found in this genus (Sysuev *et al.*, 1978). However, it is possible that in these organisms, polyP is formed using pyroP as a primer although, in those few micro-organisms in which polyP metabolism has been studied in detail, polyP is formed by phosphate adenyl kinase acting directly on orthophosphate (Wood & Clarke, 1988). We presently have insufficient information about the metabolism of inorganic phosphorus to know how polyP is formed in the genus *Phytophthora*.

If phosphonate increases the rate of synthesis of pyroP and polyP, the most logical site to examine is the mitochondria, where it has been demonstrated that proton-translocating pyrophosphatases exist (Griffiths & Halestrap, 1993) and where there is some evidence for the presence of polyP accumulation, at least in yeast (Beauvoit *et al.*, 1989). An increase in the viscosity of the inner phase of mitochondria can result in the formation of pyroP rather than ATP in some species of micro-organism (Dawes, 1986) and it has been well established that the chromatophores of *Rhodospirillum rubrum* contain a pyrophosphatase which utilizes the photosynthetically generated proton gradient in competition with the proton translocating ATPase (Mitchell *et al.*, 1972; Nyren *et al.*, 1986).

Accumulation of pyroP and polyP can be expected to have two consequences, either of which might be expected to reduce growth. Formation of pyroP and polyP from orthophosphate requires energy. This is ultimately dependent upon oxidation of the carbon source and, if growth rates are to be maintained when there is a significant diversion of energy to stored phosphoanhydride bonds, an increase in utilization of glucose (the primary carbon source) per unit mycelial mass would be required. As we have shown, there was neither an increase in glucose utilization nor an increase in respiration in the presence of phosphonate in the case of *P. palmivora*. Barchietto *et al.* (1992) had previously shown that phosphonate had no effect on oxygen consumption by *P. citrophthora* during the first 2 h of exposure, and inhibited respiration occurred after that. Increased accumulation of pyroP and polyP in the presence of phosphonate must therefore divert ATP from other pathways of metabolism, which can only result in a decrease in the growth rate.

The very large increases in pyroP might be expected to have a further effect, unless localized in a compartment isolated from the cellular metabolism. PyroP is formed by cleavage of nucleotide triphosphates in pyrophosphorylation reactions. These reactions occur in all major biosynthetic pathways, and the formation of UDPG from UDP and glucose 1-phosphate (Munch-Petersen *et al.*, 1953) is a typical example. PyroP formed during these reactions is normally hydrolysed rapidly by inorganic pyrophosphatase, regenerating orthophosphate and assisting in the formation of the dinucleotide product by a mass action effect. If the concentration of pyroP increases, pyrophosphorylation reactions produce lower concentra-

tions of their products which, like UDPG, are key intermediates in synthesis of polysaccharides, nucleic acids, lipids and proteins. Therefore a wide range of metabolic pathways essential to growth would be reduced in activity as pyroP accumulates.

It was reported (Barchietto *et al.*, 1992) that exposure of *P. citrophthora* to phosphonate for 24 h resulted in a tenfold increase in the extractable activity of UDPG pyrophosphorylase. One interpretation of this result might be that the increase in the amount of enzyme present is the result of a feed-back loop, working to maintain the level of UDPG in the presence of phosphonate. We do not know whether the effect of pyroP accumulation can be compensated for in all pyrophosphorylase reactions and unless it can be, inhibition of growth must result.

Accumulation of polyP and pyroP will also be expected to alter the concentration of cations such as potassium, magnesium, calcium and iron, all of which have high affinities for pyroP and polyP. This alteration in internal ion balance might also influence the activity of a range of enzymes catalysing essential steps in metabolism.

If the explanation of phosphonate toxicity lies in its capacity to increase pyroP and thus indirectly inhibit pyrophosphorylase reactions, it remains to be explained why higher plants and animals should be so insensitive to the presence of high concentrations of phosphonate.

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