Alterations in nucleotide and pyrophosphate levels in *Phytophthora palmivora* following exposure to the antifungal agent potassium phosphonate (phosphite)

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Short exposure (up to 3 h) of phosphate-starved mycelium from *Phytophthora palmivora* to the antifungal agent potassium phosphonate resulted in decreased levels of NAD, ATP, and a number of compounds tentatively identified as polyphosphorylated nucleotides ('alarmones'). ADP, AMP and adenosine levels were not increased, as would be expected if phosphorylation were the site of inhibition. Pyrophosphate levels, however, were raised. The incorporation of [³²P]phosphate into phospholipids and other macromolecules was not affected during this short exposure. Together, these results suggest that adenylate synthesis may be a primary site of action of phosphonate in the fungus.

Introduction

Diseases of plants caused by fungi belonging to the class Oomycetes have proved difficult to control by chemical means. Recently, a number of fungicides have been developed which are effective against these fungi (see reviews by Cohen & Coffey, 1986; Schwinn & Urech, 1986; Schwinn & Staub, 1987). One of these was aluminium ethylphosphonate (Aliette, syn. Fosetyl-Al produced by Rhone-Poulenc LA; Williams *et al.*, 1977). Subsequently, aluminium ethylphosphonate was found to be degraded rapidly in soil and plant tissue to ethanol and phosphonate (Cohen & Coffey, 1986. [The term phosphorous acid (phosphite) has been widely used as a synonym for phosphonic acid (Ouimette & Coffey, 1989).] Phosphonate is now accepted as the active component of aluminium ethylphosphonate. It is as effective as aluminium ethylphosphonate in controlling several plant diseases in the field (Pegg *et al.*, 1985; Rohrbach & Schenck, 1985; Walker, 1988; Ouimette & Coffey, 1989).

The mode of action of phosphonate in susceptible fungi is not known. Phosphonates appear to be fungistatic rather than fungicidal (Schwinn & Staub, 1987) and the action of plant defences may be necessary to achieve complete control in the host-parasite situation (Smillie *et*

al., 1989a). Alterations of the fungal surface are induced by phosphonate (R. H. Dunstan, personal communication) and may enable the plant to mount a more effective defence response (Saindrenan *et al.*, 1988; Smillie *et al.*, 1989b; Dercks & Creaser, 1989). Studies to date show that phosphonate has a wide range of effects on susceptible fungi. Mycelia grown in its presence are severely deformed (Abu-jawdah, 1983; Dercks & Buchenauer, 1987; J. M. Griffith & B. R. Grant, unpublished), and numbers of sporangia and oospores are reduced (Coffey & Joseph, 1985). Cysts derived from phosphonate-grown sporangia produce severely stunted germ-tubes on germination (J. M. Griffith, unpublished). Dercks & Buchenauer (1987) found that aluminium ethylphosphonate disturbed cell wall synthesis and phospholipid metabolism. In our laboratory, ³¹P-NMR studies have shown that phosphonate treatment of *Phytophthora palmivora* resulted in marked alterations in the distribution of several phosphorus components, including a reduction in the chain length of polyphosphates (Niere *et al.*, 1990); R. H. Dunstan (personal communication), using GC-mass spectroscopy, has found perturbations in lipid composition, cell wall material and a number of intermediary metabolites in *P. palmivora* growing in the presence of sub-toxic concentrations of phosphonate. In this paper, we report the effects of short-term exposure to phosphonate on the metabolism of this organism.

A preliminary report of part of this work was presented to the Mediterranean Phytopathological

Abbreviations: LPR, low-phosphate medium; OPR, zero-phosphate medium; PEI, polyethyleneimine; PRPP, 5'-phospho- α -D-ribose 1-pyrophosphate.

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Methods

Organism. The strain of *Phytophthora palmivora* (Butl.) Butl., ATCC 26286, and the methods used for routine culture have been described previously (Grant *et al.*, 1984, 1985).

Growth of mycelia. The media used and the conditions for the growth of the organism are described by Griffith *et al.* (1989a). Briefly, the media used were a low- P_i medium (termed 'LPR'), which contained 0.1 mM P_i , and zero-phosphate medium (termed 'OPR') containing no P_i . The fungus was grown in 50 ml LPR medium in 250 ml flasks for 7 d at 26 °C. The dry weight at harvest was approximately 30 mg per flask.

Mycelium from each flask was harvested by filtration onto glass-fibre paper (Whatman GF/C), washed with three 15 ml volumes of distilled water at room temperature, and placed in 100 ml OPR medium (diluted 1:3 with distilled water) in a 250 ml flask. Flasks were shaken at 100 oscillations min^{-1} at 26 °C. Non-radioactive P_i was added (1 μmol per flask) and the mycelium shaken for 1 h. Then, 100 μmol potassium phosphonate (pH 6.2) was added to the test flasks. Fifteen minutes later (termed 'zero time'), 5 μmol of $^{32}P_i$ (specific activity approx. 120 TBq mol^{-1}) was added to both control and test flasks. The mycelium was then harvested after a designated interval (generally 3 h later).

Extraction of mycelium. (a) *Cold perchloric acid (PCA) extraction.* Mycelia, washed as described above, were snap-frozen in liquid nitrogen. Ten millilitres of ice-cold 7% (v/v) PCA was added and the sample held at -20 °C for approximately 1 h. It was then allowed to thaw in ice, homogenized for 1 min using an Ultra-Turrax homogenizer (Janke & Kunkel, West Germany) and the mycelial fragments removed by filtration. The resulting solution was neutralized to pH 7 with KOH and passed through Whatman no. 1. filter paper to remove the KClO_4 precipitate. The filtrate was freeze-dried, redissolved in water, and a further batch of KClO_4 crystals removed by centrifugation. The latter procedure was repeated and the filtrate made to a known final volume (0.5 or 1.0 ml). Samples were stored at -20 °C prior to assay.

(b) *Cold formic acid extraction.* The washed mycelia were frozen in liquid nitrogen. Then 10 ml ice-cold 1 M-formic acid (Payne & Ames, 1982) was added to each sample, and the samples held at -20 °C for 1 h. The samples were then thawed and homogenized as described above. The mycelial fragments were removed by filtration, and the resulting solution was freeze-dried and made to a final volume of 1 ml with water.

(c) *Chloroform/methanol (lipid), KOH and hot PCA extractions.* These were done essentially as described by Niere *et al.* (1990), except that the hot PCA extraction was for 3 h at 85 °C, rather than in a boiling water bath for 1 h.

Radioactivity. The amount of radioactive phosphorus in the various fractions was determined either by Cerenkov counting or by scintillation counting using an LKB 1217 scintillation counter.

Chemicals. Nucleotides and tetrabutylammonium hydroxide were obtained from Sigma. Phosphorous acid (phosphonate) was from Albright & Wilson (Aust.) Ltd. Methanol (liquid chromatography grade) was from Millipore (Aust.). $^{32}P_i$ was obtained from the Australian Atomic Energy Commission, Lucas Heights, NSW. It was assumed to be carrier-free and was added to non-radioactive stock solutions to give a specific activity in the range of 120 TBq mol^{-1} . [^{32}P]ATP was from Bresa Ltd. (South Australia). [^{32}P]Pyrophosphate and [^{32}P]tripolyphosphate were prepared by heating a freeze-dried sample of potassium [^{32}P]phosphate at 400 °C in a muffle furnace for 1 h.

Thin-layer chromatography. Thin-layer chromatography of cold PCA and formic acid extracts was done on poly(ethyleneimine)-cellulose (PEI-cellulose) plates containing a fluorescent indicator (Merck), using the two-dimensional system of Randerath & Randerath (1964). Chromatography of lipid extracts was done on silica gel-60 aluminium plates without a fluorescent indicator (Merck), using the two-dimensional system of Wassef & Hendrix (1977). Radioactive compounds were visualized by autoradiography (Griffith *et al.*, 1989a) and quantified using a computing densitometer model 300A (Molecular Dynamics, California, USA). Non-radioactive markers were located by UV absorption (nucleotides) or by phosphomolybdate sprays (phosphorus esters) – that of Bochner *et al.* (1981) was used for the PEI-cellulose plates and that of Rosenberg (1959) for the silica gel plates. Phosphatidylcholine, -ethanolamine, -serine and -inositol, and 'ceramide-AEP' were identified by their colour reaction with specific sprays (Kates, 1982) and their positions on chromatography (Hendrix & Rouser, 1976; Wassef & Hendrix, 1977; Kates, 1982; Renkonen & Luukkonen, 1976). The sprays used were Dragendorff, ninhydrin and orcinol reagents. A number of minor spots were not identified.

Paper electrophoresis. Electrophoresis and autoradiography were done as described by Griffith *et al.* (1989a).

High-pressure liquid chromatography (HPLC). This was done using two reverse-phase columns in series (100 mm and 50 mm in length, 4 mm internal diameter, 5 μm particle size; Scientific Glass Engineering Ltd, Aust.). Compounds were detected by changes in UV absorbance at 254 nm using a Beckman model 160 absorbance detector and radioactivity was measured using a LKB-Wallac 1208 Betacord radioactivity monitor. Gradients were generated using two Waters M-45 solvent delivery systems and a model 660 solvent programmer. Generally, the solvent delivery programme no. 5 was used. Data were acquired and analysed using a chromatography program (DAPA Scientific Software, Australia). Solvent A consisted of 4 mM-tetrabutyl ammonium hydroxide in 0.1 M- KH_2PO_4 buffer, pH 6.00. Solvent B consisted of 50% (v/v) methanol and 50% (v/v) 0.1 M- KH_2PO_4 , pH 6.00. The flow rate was 1 ml min^{-1} . Generally programme 5 was used, and the volume of samples analysed was 20 μl . The optimal chromatographic conditions varied slightly between different experiments. The actual conditions used are indicated in the text.

Results

Distribution of phosphorus-labelled material in mycelium

P_i deprivation was used to introduce high levels of phosphonate rapidly into *P. palmivora* (Griffith *et al.*, 1989a, b). Its effect on phosphorus metabolism was then studied over a period of several hours.

Initial results, using P_i -deprived mycelium (Griffith *et al.*, 1989a), were variable. In some cases, addition of phosphonate caused such a severe depression in phosphorus metabolism that very little $^{32}P_i$ was assimilated. Consistent results were obtained when a small amount of non-labelled P_i (1 μmol) was added to the fungus 1 h before the addition of 100 μmol phosphonate. (Preliminary studies showed that most of the P_i had been taken up by the time the $^{32}P_i$ was added.) After the addition of phosphonate, the fungus was shaken for a further 15 min before the $^{32}P_i$ (5 μmol) was added. P_i concentrations

Table 1. ^{32}P distribution in mycelium of *P. palmivora*

Mycelia from eight flasks of LPR medium were treated in separate flasks for 1 h with 1 μmol non-labelled P_i . Half were then given 100 μmol phosphonate for 15 min, then 5 μmol $^{32}\text{P}_i$ was added to all flasks. After 3 h, the mycelia were harvested, washed, and frozen in liquid N_2 . The mycelia were extracted with ice-cold PCA, then chloroform/methanol, 0.1 M-KOH, and hot PCA. The distribution of the ^{32}P in the various fractions was then determined. The results are the means of four flasks, $\pm$ SEM.

Fraction	^{32}P recovered (nmol)	
	Control	Phosphonate treated
Cold PCA	540.3 $\pm$ 152	488.2 $\pm$ 145
Lipid	59.1 $\pm$ 22.0	56.7 $\pm$ 9.5
0.1 M-KOH	105.0 $\pm$ 28.3	85.4 $\pm$ 13.4
Hot PCA	28.2 $\pm$ 6.9	23.0 $\pm$ 4.1
Pellet	2.3 $\pm$ 0.6	1.5 $\pm$ 0.3
Total in mycelium	734.9 $\pm$ 202.6	654.8 $\pm$ 167.5
Remaining in medium	3270 $\pm$ 435	3400 $\pm$ 187
Recovery	80.1%	81.1%

Table 2. ^{32}P distribution in lipids from *P. palmivora*

Phospholipids, prepared from the mycelia used in the experiment shown in Table 1, were separated on silica gel plates using a two-dimensional system. Autoradiographs were prepared and the spots were quantified using a densitometer. The results, in arbitrary units, are the means of four separate extracts of mycelium, $\pm$ SEM.

Phospholipid	Control	Phosphonate treated
Phosphatidic acid	7.1 $\pm$ 3.8	5.9 $\pm$ 4.3
Phosphatidyl-ethanolamine	7.8 $\pm$ 1.8	7.9 $\pm$ 1.1
Phosphatidyl-serine and -inositol	5.2 $\pm$ 2.6	6.4 $\pm$ 2.6
Ceramide-AEP	8.4 $\pm$ 2.5	7.5 $\pm$ 1.8
Phosphatidylcholine	10.7 $\pm$ 2.2	11.1 $\pm$ 1.6
Unknown 1	0.6 $\pm$ 0.3	0.3 $\pm$ 0.2
Unknown 2	1.2 $\pm$ 0.8	1.2 $\pm$ 1.6
Unknown 3	1.6 $\pm$ 1.8	1.6 $\pm$ 1.7

* This compound has been identified as a phosphonolipid (Wassef & Hendrix, 1977; Creamer & Bostock, 1986), but ^{31}P -NMR studies have failed to reveal any peaks corresponding to phosphonolipids (Niere *et al.*, 1990).

were maintained at low levels, since micromolar concentrations of P_i significantly inhibit phosphonate uptake in this strain of *P. palmivora* (Griffith *et al.*, 1989b; cf. Barchietto *et al.*, 1988, 1989).

Three hours after the addition of the $^{32}\text{P}_i$, phosphonate had not altered the distribution of ^{32}P between the major fractions extracted from the mycelium (Table 1). Further fractionation of the phospholipid components failed to

reveal any major differences between the treated and the non-treated samples (Table 2). However, paper electrophoresis of the cold PCA extract showed that some bands were reduced in the phosphonate-treated samples (data not shown).

Reverse-phase chromatography of acid-soluble material

The PCA extracts were further fractionated using a reverse-phase HPLC system. The results of a typical experiment are shown in Figs 1–3. Fig. 1 shows the UV absorbance profile of material at 'zero time', just before the addition of $^{32}\text{P}_i$. At this stage (15 min exposure to phosphonate), there were no detectable differences between the phosphonate-treated material and the control. However, after 3 h incubation in the presence of $^{32}\text{P}_i$, there was a marked reduction in the concentration of compounds running in the positions of ATP and of NAD (Figs 2 and 3). The concentrations of a number of compounds eluting near ATP were also reduced. Although these compounds were highly labelled (Fig. 3), their UV absorbance was low compared with ATP (Fig. 2). A ^{32}P -labelled peak eluting just after P_i also increased

Fig. 1. UV absorbance profile of PCA extracts from *P. palmivora* mycelium harvested at 'zero time'. Two samples of mycelia from phosphorus-deficient medium were treated with 1 μmol non-radioactive P_i for 1 h. Potassium phosphonate (100 μmol) was then added to one of the samples, and after a further 15 min both samples were harvested ('zero time'). PCA extracts were prepared and the material fractionated on HPLC (15 min run, 40% solvent B). (a) Control; (b) phosphonate-treated. The identity of the markers indicated by arrows is: 1, NAD; 2, adenosine/AMP; 3, ADP; 4, ATP.

Fig. 2. UV absorbance profile of PCA extracts from *P. palmivora* mycelium harvested 3 h after the addition of $^{32}\text{P}_i$. Conditions were as described for Fig. 1, except that $5\text{ }\mu\text{mol } ^{32}\text{P}_i$ was added at 'zero time'. (a) Control; (b) phosphonate-treated. The arrows indicate the positions of the following compounds: 1, NAD; 2, adenosine/AMP; 3, ADP; 4, not identified; 5, ATP. The compounds tentatively identified as polyphosphorylated nucleotides ('alarmones') are indicated with an asterisk.

after phosphonate treatment (Fig. 3b). This peak had little, if any, UV absorbance.

The positions of NAD, ADP and ATP were confirmed by spiking the extracts with authentic nucleotides (including ^{32}P ATP). The concentrations of NAD and ATP in the phosphonate-treated material were about half those of the control (data not shown). In another experiment using $^{32}\text{P}_i$ of higher specific activity (approx. 400 TBq mol^{-1}), differences in NAD and ATP levels could be detected as early as 30 min after the addition of $^{32}\text{P}_i$.

Charcoal-treatment of the PCA extracts at acid pH resulted in the disappearance of almost all of the UV-absorbing material (Fig. 4a, c), including the peaks with the high level of radioactivity and low UV absorbance (Fig. 4b, d). Based on this observation and on their position in two-dimensional thin-layer chromatography (see below), these compounds have been tentatively identified as polyphosphorylated nucleotides (see Discussion). Formic acid has been used to extract these types of compounds from bacteria (Payne & Ames, 1982).

Fig. 3. ^{32}P profile of PCA extracts from *P. palmivora* mycelium harvested 3 h after the addition of $^{32}\text{P}_i$. This profile was determined concurrently with the UV absorbance profile presented in Fig. 2, and has been adjusted for the time difference between the UV and radioactivity monitors. (a) Control; (b) phosphonate-treated. The arrows indicate the positions of the following compounds: 1, P_i ; 2, pyrophosphate; 3, NAD (determined from Fig. 2); 4, tripolyphosphate; 5, 6, not identified; 7, ATP. The compounds tentatively identified as polyphosphorylated nucleotides ('alarmones') are indicated with an asterisk.

However, we did not find these compounds in formic acid extracts of *P. palmivora*.

A number of radioactive compounds, however, were not adsorbed by charcoal (Fig. 4d). As shown in Fig. 3, peaks 1, 2 and 4 co-chromatographed with P_i , ^{32}P pyrophosphate and ^{32}P tripolyphosphate respectively. It seems probable that some of the other unidentified peaks are higher polyphosphates, since these are known to be present in this organism (Niere *et al.*, 1990).

Two-dimensional thin-layer chromatography of acid-soluble material

PCA- and formic-acid-extracted material was chromatographed on PEI-cellulose plates. It was possible to identify most of the spots by co-chromatography with known standards. Charcoal-treatment resulted in the disappearance of those spots tentatively identified as nucleotides. Similarly, heat-treatment (10 min in 1 M-HCl at $100\text{ }^\circ\text{C}$) resulted in hydrolysis of all compounds, except that identified as fructose 1,6-bisphosphate, which was reduced in intensity, and a small spot

Fig. 4. UV absorbance and ^{32}P profiles of PCA extracts from *P. palmivora* mycelium before and after charcoal-treatment. PCA extracts were prepared from mycelium incubated in the presence of $5\ \mu\text{mol } ^{32}\text{P}_i$, as described in the text. A 0.2 ml portion of the material was acidified by the addition of an equal volume of 5% (v/v) formic acid, and charcoal (approx. 10 mg) was added. The charcoal was removed by centrifugation (Microfuge, 2 min at 10000 r.p.m.) followed by filtration through a $0.45\ \mu\text{m}$ membrane (Millipore). The extract was then fractionated by HPLC and the UV absorbance and ^{32}P profiles determined. Chromatographic conditions: 15 min run, 35% solvent B. (a, b) Material before charcoal-treatment: (a) UV absorbance profile; (b) ^{32}P profile. (c, d) Material after charcoal-treatment: (c) UV absorbance profile; (d) ^{32}P profile. Note: The data for (c) and (d) have been corrected for dilution; the new peak at the solvent front in (c) results from the added formic acid.

migrating in the region of α -glycerophosphate (cf. Nelson & Kornberg, 1970). Exposure to phosphonate resulted in a reduction in the levels of ATP, of material running in the region of polyphosphates (Lusby & McLaughlin, 1980) and the polyphosphorylated nucleotides (Cashel & Gallant, 1969), and of an unidentified spot which migrated at the solvent front. Fructose 1,6-bisphosphate was present at approximately the same level in both treated and control material. The concentration of pyrophosphate, however, had almost doubled in phosphonate-treated material. It was not possible to identify precisely which spot corresponded to NAD, as this compound co-chromatographed with α -glycerophosphate. It was clear, however, that the amount of radioactivity migrating in the region of NAD was very low. Subsequent fractionation of these extracts on HPLC using a shallower gradient (programme no. 6) revealed

that there were several radioactive compounds eluting in the region of NAD (data not shown). These peaks may include fructose 1,6-bisphosphate.

Discussion

The present work indicates that, initially, the addition of phosphonate to *P. palmivora* mycelium alters the synthesis of only a few phosphorus-containing compounds. The earliest effects we have observed are a decrease in the levels of NAD, ATP and other polyphosphorylated nucleotides, and an increase in pyrophosphate. Differences in nucleotide levels were detected as early as 30 min after phosphate addition. The common link between adenylate synthesis and NAD synthesis is 5'-phospho- α -D-ribose 1-pyrophosphate (PRPP). PRPP is synthesized from ribose 5'-phosphate and ATP. It is a key intermediate in the biosynthesis of purines and pyrimidines, as well as histidine and tryptophan (Stryer, 1981). Phosphonate may act directly on PRPP synthetase as a tight binding inhibitor. Alternatively, phosphonate may act on PRPP synthetase indirectly. For instance, a deficiency of pyruvate kinase in red blood cells results in lowered ATP and raised 2,3-phosphoglycerate levels (Tanaka & Paglia, 1971). One would expect these cells to accumulate AMP and ADP as a result of their inability to regenerate ATP. Instead, the levels of all adenine nucleotides and of total NAD are decreased (Tanaka & Paglia, 1971). The dissociation of PRPP synthetase is promoted by 2,3-phosphoglycerate, as well as by lowered ATP levels (Becker *et al.*, 1977). Zerez *et al.* (1986) have suggested that it is the raised 2,3-phosphoglycerate concentrations, rather than the lowered ATP levels, which are the major cause of the observed decrease in the adenine nucleotide pool *in vivo*. Regardless of the mechanism, inhibition at this site would reduce the pool sizes of adenylate nucleotides and NAD, and might be expected to have numerous effects on intermediary metabolism.

It is clear, however, that the effects of phosphonate on metabolism are relatively slow. Initially, the substitution of pyrophosphate or polyphosphate for ATP as a source of phosphoryl groups may minimize the effect of phosphonate on the ATP pool size (Harold, 1966; Kulaev & Vagabov, 1983; Wood, 1985; Wood & Clark, 1988). The observation that the levels of fructose 1,6-bisphosphate did not alter significantly in the presence of phosphonate indicates that the early steps of glycolysis are not completely blocked. The raised pyrophosphate concentrations observed may result from either the inhibition of an as yet unidentified enzymic reaction, or the increased catabolism of tri- and higher polyphosphates. The latter explanation is compatible with the

decrease in the average chain length of polyphosphate observed by Niere *et al.* (1990).

There was no increase in the pool sizes of ADP, AMP or adenosine as the pool size of ATP decreased. This indicates that the phosphorylation mechanism is intact in the presence of phosphonate. Further evidence for this interpretation is provided by the failure of phosphonate to alter phospholipid synthesis at this early stage. The pools of C₅ and C₆ sugars and sugar alcohols are also unchanged in the presence of phosphonate (R. H. Dunstan, personal communication).

We have found a number of compounds in the PCA extracts of P_i-starved mycelium which we have tentatively identified as polyphosphorylated nucleotides. These unusual nucleotides, colloquially known as 'alarmones', may act as signals for regulation (Rapaport & Zamecnik, 1976; Zamecnik, 1983) and alert the cell to the onset of a particular stress (Bochner, 1987). Diadenosine 5',5'''P₁,P₄-tetrphosphate (AppppA), for instance, has been shown to activate DNA polymerase in HeLa cells (Rapaport *et al.*, 1981). We are not aware of any reports of the effects of P_i-deprivation on the synthesis of alarmones in lower eukaryotes; however, P_i-deficiency did not produce increased levels of AppppA in the bacterium *Salmonella typhimurium* (Bochner *et al.*, 1984). The dramatic reduction of the levels of the alarmones in the presence of phosphonate reported here may either be the result of a direct inhibition of the enzymes involved in their biosynthesis, or an indirect result of the reduction in adenylate pool size.

In conclusion, these results point to a specific site of action of phosphonate in *P. palmivora* – that of the synthesis of adenylate and a further possible site at the synthesis of the alarmones. The decreased level of ATP (but not energy charge) as a consequence of adenylate depletion may be minimized by the ability of the organism to use, in some reactions, polyphosphates rather than ATP as a source of high-energy phosphate bonds. However, as adenylate pool sizes are lowered, it could be anticipated that many synthetic reactions would be altered, accounting for the changes in the cell wall and cell surface polysaccharides already observed.

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