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

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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 $^{32}$P 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-AI 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 (phosphate) 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, $^{31}$P-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
Union Conference at Palermo, Sicily, April, 1989 (Smillie et al., 1989b).

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 were a low-P medium (termed 'LPR'), which contained 0.1 mM Pi, and a zero-phosphate medium (termed '0PR') containing no Pi. 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 0PR 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 Pi was added (1 μmol per flask) and the mycelium shaken for 1 h. Then, 100 μmol potassium phosphate (pH 6.2) was added to the test flasks. Fifteen minutes later (termed 'zero time'), 5 μmol of ³²Pi (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 KClO₄ precipitate. The filtrate was freeze-dried, redissolved in water, and a further batch of KClO₄ crystals removed by centrifugation. The latter procedure was repeated and the filtrate made to a final known 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 scintillator counter.

Chemicals. Nucleotides and tetrabutylammonium hydroxide were obtained from Sigma. Phosphoric acid (phosphonate) was from Albright & Wilson (Aust.) Ltd. Methanol (liquid chromatography grade) was from Millipore (Aust.). ³²P, 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 [³²P]ADP and [³²P]ATP was from British Nuclear Fuels, Ltd. South Australia. [³²P]Pyrophosphate and [³²P]triphosphate were prepared by heating a freeze-dried sample of potassium [³²P]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, 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 Dragendorf, 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-tetra-butyl ammonium hydroxide in 0.1 M-KH₂PO₄ buffer, pH 6.00. Solvent B consisted of 50% (v/v) methanol and 50% (v/v) 0.1 M-KH₂PO₄, 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

Pᵢ 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 Pᵢ-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 ³²Pᵢ was assimilated. Consistent results were obtained when a small amount of non-labelled Pᵢ (1 μmol) was added to the fungus 1 h before the addition of 100 μmol phosphonate. (Preliminary studies showed that most of the Pᵢ had been taken up by the time the [³²P]ᵢ was added.) After the addition of phosphonate, the fungus was shaken for a further 15 min before the [³²P]ᵢ (5 μmol) was added. Pᵢ concentrations...
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 32P_i, phosphonate had not altered the distribution of 32P 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 32P_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 32P_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 32P-labelled peak eluting just after P_i also increased.

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**Table 1. 32P distribution in mycelium of P. palmivora**

Myelia 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 32P_i was added to all flasks. After 3 h, the myelia were harvested, washed, and frozen in liquid N_2. The myelia were extracted with ice-cold PCA, then chloroform/methanol, 0-1 M-KOH, and hot PCA. The distribution of the 32P in the various fractions was then determined. The results are the means of four flasks, ± SEM.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Phosphonate treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold PCA</td>
<td>546.3 ± 152</td>
<td>488.2 ± 145</td>
</tr>
<tr>
<td>Lipid</td>
<td>59.1 ± 22.0</td>
<td>56.7 ± 9.5</td>
</tr>
<tr>
<td>0-1 M-KOH</td>
<td>105.0 ± 28.3</td>
<td>85.4 ± 13.4</td>
</tr>
<tr>
<td>Hot PCA</td>
<td>28.2 ± 6.9</td>
<td>23.0 ± 4.1</td>
</tr>
<tr>
<td>Pellet</td>
<td>2.3 ± 0.6</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Total in mycelium</td>
<td>734.9 ± 202.6</td>
<td>654.8 ± 167.5</td>
</tr>
<tr>
<td>Remaining in medium</td>
<td>3270 ± 435</td>
<td>3400 ± 187</td>
</tr>
<tr>
<td>Recovery</td>
<td>80.1%</td>
<td>81.1%</td>
</tr>
</tbody>
</table>

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**Table 2. 32P distribution in lipids from P. palmivora**

Phospholipids, prepared from the myelia 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, ± SEM.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>Phosphonate treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidic acid</td>
<td>7.1 ± 3.8</td>
<td>5.9 ± 4.3</td>
</tr>
<tr>
<td>Phosphatidyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethanolamine serine</td>
<td>7.8 ± 1.8</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>and -inositol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Ceramide-AEP*’</td>
<td>5.2 ± 2.6</td>
<td>6.4 ± 2.6</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>8.4 ± 2.5</td>
<td>7.5 ± 1.8</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>0.6 ± 0.3</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>1.2 ± 0.8</td>
<td>1.2 ± 1.6</td>
</tr>
<tr>
<td>Unknown 3</td>
<td>1.6 ± 1.8</td>
<td>1.6 ± 1.7</td>
</tr>
</tbody>
</table>

* This compound has been identified as a phosphonolipid (Wassef & Hendrix, 1977; Creamer & Bostock, 1986), but 31P-NMR studies have failed to reveal any peaks corresponding to phosphonolipids (Niere et al., 1990).
Fig. 2. UV absorbance profile of PCA extracts from *P. palmivora* mycelium harvested 3 h after the addition of $^{32}$P. Conditions were as described for Fig. 1, except that 5 μmol $^{32}$P 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.

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°C) resulted in hydrolysis of all compounds, except that identified as fructose 1,6-bisphosphate, which was reduced in intensity, and a small spot
fractionated by HPLC and the UV absorbance and $^{32}$P profiles determined. Chromatographic conditions: 15 min run, 35% solvent through a 0.45 pm membrane (Millipore). The extract was then 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 μm 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 phosphonate 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 phosphate. Further evidence for this interpretation is provided by the failure of phosphate 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 phosphate (R. H. Dunstan, personal communication).

We have found a number of compounds in the PCA extracts of P₃-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''P₄, P₄-tetraphosphate (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 P₃-deprivation on the synthesis of alarmones in lower eukaryotes; however, P₃-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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References


