³¹P NMR studies on the effect of phosphite on *Phytophthora palmivora*

JULIE O. NIERE,¹* JULIA M. GRIFFITH² and BRUCE R. GRANT²

¹Department of Applied Chemistry, Phillip Institute of Technology, Bundoora, Victoria, Australia

²School of Biochemistry, University of Melbourne, Parkville, Australia

(Received 14 April 1989; revised 4 August 1989; accepted 21 September 1989)

³¹P NMR spectra were obtained from perchloric acid (PCA) and KOH extracts of *Phytophthora palmivora* mycelium. Signals indicating the presence of large amounts of short-chain polyphosphate were observed in the spectra of PCA extracts of mycelia grown under both low (0.1 mM) and high (10 mM) phosphate conditions. The mean chain length of polyphosphate was calculated from the relative areas of signals arising from terminal and internal P nuclei in the polyphosphate chain. The small amount of polyphosphate evident in the KOH extract had an average chain length similar to PCA-soluble polyphosphate. ³²P tracer studies indicated that phosphorus in the PCA fraction accounted for between 50 and 60% of total phosphorus, the bulk of the remainder being divided between the lipid and KOH extracts. The presence of the fungicide phosphorous acid markedly reduced the average chain length of acid-soluble polyphosphate. This reduction occurred both under low-phosphate conditions, in which treatment with phosphorous acid retards growth, and under high-phosphate conditions, in which no significant growth retardation is observed. Treatment with phosphorous acid perturbed phosphorus distribution and lipid composition under low-phosphate conditions.

Introduction

Phosphorous acid (phosphite) and fosetyl-Al (Aliette) act as systemic fungicides against a number of members of the genus *Phytophthora*. It has recently been shown (Cohen & Coffey, 1986) that the active toxiphore of Aliette is phosphite. Both compounds are more effective against target fungi *in planta* than *in vitro* and this has led to the proposal that Aliette and (by implication) phosphite operate via the plants' defence systems rather than through any toxic effect on the pathogen itself (Guest, 1984; Schwinn & Urech, 1986). However, work by Coffey (Coffey & Bower, 1984; Bower & Coffey, 1985; Coffey & Joseph, 1985; Dolan & Coffey, 1988) indicated that phosphite did act directly on the fungus. The mechanism of action is still not known. Severe morphological changes in the mycelia of Aliette- and phosphite-treated *Phytophthora palmivora* have been observed (Dercks & Buchenauer, 1987; J. M. Griffith, unpublished results). Studies with [³²P]phosphite indicate that the fungicide is not metabolized by *P.*

palmivora, but significant alterations in ³²P labelling of metabolites occurred (J. M. Griffith, unpublished results). Here we report a study by ³¹P NMR and ³²P labelling of the distribution of phosphorus among various mycelial fractions and chemical species in *P. palmivora* grown under low- and high-phosphate conditions in the presence and absence of phosphite.

Methods

Reproducibility. All results reported are based on at least three experiments, and in the case of ³²P studies, each experiment was carried out in duplicate.

Strains. The isolate of *P. palmivora* used was ATCC 26286. Its culture and maintenance have been described previously (Grant *et al.*, 1984).

Media and growth conditions. Ribeiro's defined medium (Ribeiro *et al.*, 1965) as modified by Fenn & Coffey (1984) was used for mycelial production, phosphate concentrations being adjusted as required. As the uptake of phosphite and the degree of growth inhibition have been shown to be sensitive to external phosphate concentrations (J. M. Griffith, unpublished results), mycelia were grown in both high-phosphate and low-phosphate media. 'Low-phosphate medium' (LPR) contained 0.1 mM-phosphate, and 'high-phosphate medium' (HPR) contained 10 mM. Where appropriate, phosphite solution (0.1 M) which had been autoclaved separately was added to the culture medium to give a final concentration of 1 mM.

Mycelia were grown in 50 ml of either LPR or HPR medium in

Abbreviations: PCA, perchloric acid; LPR, low-phosphate Ribeiro (medium); HPR, high-phosphate Ribeiro (medium); polyP, polyphosphate; tripolyP, tripolyphosphate; tetrapolyP, tetrapolyphosphate; NTP, nucleoside triphosphate; NDPG, nucleoside diphosphate glucose; CAEP, ceramide aminoethylphosphonate.

250 ml flasks. Mycelium for inoculation was propagated on LPR medium to which 2% (w/v) agar had been added. Each flask was inoculated with one piece of agar (4 mm diam.). The flasks were then incubated in the dark, without shaking, for 7 d at 26 °C.

Harvesting and extraction procedures. Mycelia from 10 flasks (NMR studies) or two flasks (^{32}P studies) were collected by vacuum filtration on Whatman GF/A glass-fibre filters, washed with ice-cold water (5 × 30 ml), weighed and freeze-dried. After harvest, mycelia were first extracted with cold perchloric acid (PCA-soluble material), then with chloroform/methanol (lipid fraction) and finally with dilute KOH to obtain nucleic acid, proteinaceous material and any acid-insoluble polyphosphate. ^{32}P tracer studies were conducted concurrently to determine the fraction of total mycelial phosphorus present in each extract.

PCA extraction. The PCA extraction procedure was similar to that described by Navon *et al.* (1979). Ice-cold PCA (35%, w/v, 2 ml) was added to freeze-dried mycelium (typically 350 mg) suspended in 8 ml ice-cold water. The mixture was homogenized, freeze-thawed once in liquid nitrogen, then filtered through a sintered glass funnel. The mycelial residue was then rehomogenized in 10 ml ice-cold water, filtered and washed with water (2 × 5 ml). The combined filtrates were immediately neutralized with 6 M-KOH. Care was taken throughout to maintain the temperature of both filtrate and mycelium below 5 °C. The supernatant was removed from the potassium perchlorate precipitate and freeze-dried. The stability of polyphosphate (polyP) under acid extraction conditions was investigated by subjecting (a) an aqueous solution of synthetic polyP (Sigma PolyP type 15) and (b) a mixture of synthetic polyP solution and mycelium to the extraction procedure described above.

Lipid extraction. After PCA extraction, the mycelial residue was homogenized in chloroform/methanol (2:1, v/v, 15 ml) and filtered. The mycelial residue was rehomogenized in chloroform/methanol (15 ml), filtered and washed with the same solvent (2 × 5 ml). For NMR studies, the combined filtrates were washed once with 0.1 M-EDTA (10 ml, pH 7.5). The solvent was then removed under reduced pressure and the residue freeze-dried.

KOH extraction. The mycelial residue remaining after lipid extraction was suspended in 0.1 M-KOH (10 ml) for 3 h at room temperature. It was then homogenized and filtered. This process was repeated on the mycelial residue, which was finally washed with water (2 × 5 ml). The combined filtrates were neutralized with 1 M-HCl and freeze-dried.

Hot PCA extraction (^{32}P studies only). The mycelial residue remaining after KOH extraction was boiled in PCA (15 ml, 35% w/v) for 1 h. The mixture was filtered, the residue washed with water (2 × 10 ml) and the combined filtrates neutralized with 6 M-KOH. The resulting potassium perchlorate precipitate was removed by filtration and washed with water (3 × 2 ml). The combined filtrates were concentrated and an aliquot was taken for counting.

NMR preparation, conditions and calculations. Freeze-dried PCA or KOH 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 between 7.8 and 8.0 with 0.1 M-KOH. The final volume of the sample was adjusted to 2.00 ± 0.05 ml. Freeze-dried lipid samples were dissolved in deuteriochloroform (2 ml). All ^{31}P NMR experiments were conducted on a Bruker AM300 instrument (operating frequency 121 MHz) at a temperature of 295 ± 1 K. Spectra were normally accumulated using 45° pulses and either 0.6 s or 7 s recycle times. While producing good

signal-to-noise ratios within a reasonable time (typically 1–2 h), the shorter recycle time did not permit total relaxation of all nuclei between pulses. Moreover, peak areas under these conditions did not reflect relative concentrations of each type of nucleus due to differences in degree of saturation. However, saturation factors for all signals of interest were calculated by comparing relative peak areas obtained using 0.6 s recycle times with those obtained under conditions ensuring complete relaxation of all phosphorus nuclei (recycle time 7 s). Examples of saturation factors are: pyrophosphate, 1.0; terminal P of tripolyphosphate (tripolyP), 1.4; high-field non-terminal P of polyP, 1.4; α -P of NTP, 1.5.

The spectral width of 8300 Hz was represented by 8K data points. Broad-band proton decoupling had no effect on the linewidths of peaks arising from phosphates and was therefore only used in cases where the presence of phosphonates or phosphonolipids was known or being investigated. For example, once it had been established that no peaks were appearing in the C–P region of PCA-extract spectra, these spectra were normally run fully coupled.

Chemical shifts were referenced to a phosphocreatine (0.1 mM in a 1 mm concentric capillary tube) signal set at 2.35 p.p.m. downfield from 85% H_3PO_4 . Peaks were assigned by reference to published chemical shifts (Navon *et al.*, 1979) which, at pH 7.55, are as follows: +5.1 to +4.0 p.p.m. (fructose 1,6-bisphosphate, glucose 6-phosphate, fructose 6-phosphate); +3.0 p.p.m. (phosphate), –4.5 to –5.0 p.p.m. (terminal P on polyP > P_3); –5.2 p.p.m. (ATP γ); –5.7 p.p.m. (ADP β); –5.9 p.p.m. (terminal P in tripolyP); –6.8 p.p.m. (pyrophosphate); –9.8 p.p.m. (ADP α); –10.5 p.p.m. (ATP α); –10.6 and –12.0 p.p.m. (UDPG); –10.8 p.p.m. (NAD); –20 p.p.m. (penultimate P in polyP > P_3); –20.1 p.p.m. (inner P in tripolyP); –20.8 p.p.m. (ATP β); –21.0 p.p.m. (inner P in polyP > P_3). In cases where chemical shift was dependent on pH and metal ion concentration, assignment was checked by spiking the extract with authentic samples and varying the pH.

The proportion of total extract phosphorus (excluding phosphite) existing as inorganic polyP was calculated by dividing the combined areas of the –4.5 to –7 p.p.m. and –18 to –23 p.p.m. regions by the total area of all signals in the spectrum (excluding phosphite and phosphocreatine peaks). Allowance was made for contributions to the polyP regions by γ - and β -phosphorus nuclei of nucleoside di- and triphosphates.

The mean polyP chain length was calculated according to the following formula:

$$\text{Mean chain length} = 2 + [2(A_N/A_T)]$$

where A_N = area of non-terminal polyP region (–4.5 to –7 p.p.m.), and A_T = area of terminal polyP region (–18 to –23 p.p.m.). Again, allowance was made for contributions to these areas by nucleoside di- and triphosphates.

^{32}P studies. ^{32}P -phosphate distribution studies were carried out on 7-d-old mycelium grown in either HPR or LPR medium. Carrier-free ^{32}P -phosphate was added to the growth medium prior to autoclaving. ^{32}P content was determined by counting aliquots of each fraction in a liquid scintillation counter.

Total mycelial P was determined by counting a sample of the freeze-dried mycelium dissolved in Protosol[®] (New England Nuclear).

Thin-layer chromatography. Lipid extracts were subjected to two-dimensional thin-layer chromatography (Hendrix & Rouser, 1976) on pre-coated Merck Kieselgel 60 plates. The chromatogram was sprayed with molybdenum solution (Dittmer & Lester, 1964) and heated to differentiate phospholipid and phosphonolipid spots. After cooling, phosphonate spots remained blue while phosphate spots turned brown (Stillway & Harmon, 1980).

Fig. 1. ^{31}P NMR spectra at 121 MHz of PCA extract of *P. palmivora* (a) grown in LPR medium; (b) grown in LPR medium in the presence of 1 mM-phosphite. The peak assignments are: 1, phosphorous acid; 2, phosphomonoesters, e.g. fructose 6-phosphate, glycerophosphate, etc. (individual resonances were not assigned); 3, inorganic phosphate; 4, phosphocreatine (external standard); 5, terminal P of polyP 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, pyrophosphate; 9, $\alpha\text{-P}$ of ATP and other nucleoside tri- and diphosphates, P of NDPG, and perhaps P of NAD; 10, P of NDPG; 11, non-terminal P of inorganic polyP and $\beta\text{-P}$ of nucleoside triphosphates.

Results

Treatment of *P. palmivora* with phosphite under low-phosphate conditions resulted in an average reduction of 50% in mycelial dry weight. In contrast, treatment with phosphite under high phosphate conditions produced no significant changes in mycelial dry weight.

NMR studies

(a) *PCA extracts.* ^{31}P NMR spectra of PCA extracts of mycelia grown in LPR medium with and without phosphite are shown in Fig. 1. Large amounts of polyP were evident in the extract of the control sample (Fig. 1a), polyP phosphorus making up about 60% of total

Fig. 2. ^{31}P NMR spectra of PCA extract of *P. palmivora* (a) grown in HPR medium; (b) grown in HPR medium in the presence of 1 mM-phosphite. Peak assignments are as in Fig. 1.

PCA-soluble P. The mean length of polyP was calculated to be 4.7 ± 0.2 (mean $\pm$ SD).

The spectrum of the phosphite-treated sample is shown in Fig. 1(b). Large amounts of polyP were still present, and the fraction of PCA-soluble P existing as polyP was increased from 60 to 70%. Consistent with this change, the intensities of peaks in the phosphomonoester (+3.5 to +5.0 p.p.m.) and NTP-NDPG (−9.5 to −12.3 p.p.m.) regions decreased markedly, the signals at +4.0 p.p.m. (phosphomonoester) and −12.3 p.p.m. (NDPG)

disappearing into baseline noise. The intensity patterns of peaks in the polyP regions (−4.5 to −7 p.p.m. and −18 to −23 p.p.m.) were skewed, indicating a change in average polyP length. The mean polyP length was calculated to be 3.6 ± 0.3 , a 25% decrease compared with the control.

^{31}P NMR spectra of PCA extracts of mycelia grown in HPR medium with and without phosphite are shown in Fig. 2. Comparison of the areas of phosphite peaks with that of the phosphocreatine standard showed that in

Fig. 3. ^{31}P NMR spectrum of lipid extract of *P. palmivora* grown in HPR medium.

nearly all cases (see below for the exception) the amount of phosphite per mg dry weight of mycelium in LPR was approximately eight times that in HPR.

Under high-phosphate (HPR) conditions, treatment with phosphite resulted in a 30% drop in mean polyP chain length from 6.3 ± 0.4 to 4.3 ± 0.3 . Although no differences in levels of phosphomonoesters, phosphorylated nucleosides or NDPG were apparent, phosphite treatment usually resulted in the appearance of three small (as yet unidentified) signals at -8.5 , -8.6 and -9.0 p.p.m. In the one case where these signals did not appear, the level of phosphite was less than one-third of that normally observed in HPR samples and the decrease in mean polyP chain length was only 10% (results from this particular experiment were not included in the mean chain length calculations).

(b) *Lipid extracts.* The ^{31}P NMR spectrum of the lipid extract of LPR-grown mycelium is shown in Fig. 3. As peaks arising from phospholipids appear in a very narrow chemical shift range, considerable overlap occurs. This makes assignment difficult. However, spiking experiments indicated that the two major phospholipid components of the extract were phosphatidylethanolamine ($+0.45$ p.p.m.) and phosphatidylcholine (-0.48 p.p.m.). Treatment with phosphite produced no significant changes in the phospholipid spectra of mycelia grown in LPR or HPR media. Although the thin-layer chromatogram of the lipid extract contained a faint blue-grey spot which had migrated in a manner similar to that reported (Wassef & Hendrix, 1977) for ceramide

aminoethylphosphonate (CAEP), no peaks corresponding to phosphonolipids ($+15$ to $+23$ p.p.m.) were observed in the NMR spectra. As the presence of considerable amounts of phosphonolipid had been reported for other *Phytophthora* species (Bostock & Creamer, 1986) and *Pythium prolatum* (Wassef & Hendrix, 1977), mycelia were also extracted with 1-propanol/chloroform/methanol exactly as described by Wassef & Hendrix (1974, 1977). The NMR spectra of these extracts also showed no evidence of phosphonolipids. A mollusc (*Limax maxima* L.) known to contain phosphonates was extracted first with PCA and then with chloroform/methanol. In this case the NMR spectra of both acid and lipid extracts contained peaks typical of C-P compounds, and spraying of the two-dimensional thin-layer chromatogram of the lipid extract with molybdate reagent produced an intense blue spot which had migrated as CAEP.

(c) *KOH extracts.* The proton decoupled ^{31}P NMR spectrum of the KOH extract of HPR-grown mycelium is shown in Fig. 4. The intense absorption envelope between -1.5 and $+2$ p.p.m. can be attributed to phosphodiester presumably arising from degradation products of nucleic acid. The chemical shift of the signal appearing at $+20.2$ p.p.m. suggests that small amounts (1–2% of total KOH-soluble P) of phosphonate are present. The peaks appearing between $+4$ and $+5.5$ p.p.m. have chemical shifts characteristic of phosphomonoesters but have not yet been identified. The small peak at $+3.0$ p.p.m. is due to inorganic phosphate, part

Fig. 4. ^{31}P NMR spectrum of KOH extract of *P. palmivora* grown in HPR medium.

Table 1. Amount of phosphorus (excluding phosphite-P) in different extracts of 7-d-old *P. palmivora* mycelium

The results represent (a) mmol P per sample (mycelium from 10 flasks), and (b) mmol P (mg mycelial dry wt) $^{-1}$. Standard deviations are shown in parentheses.

Growth medium	Cold PCA soluble	Lipid	KOH soluble	Hot PCA soluble	Total mycelial P
LPR	(a) 2.1 (0.2) (b) 0.06 (0.01)	1.2 (0.5) 0.03 (0.01)	1.4 (0.2) 0.04 (<0.01)	0.1 (0.1) 0.01 (0.01)	4.8 (0.6) 0.14 (0.02)
LPR + phosphite	(a) 2.9 (0.2) (b) 0.15 (0.01)	0.9 (0.3) 0.05 (0.02)	0.8 (<0.1) 0.04 (<0.01)	0.3 (0.3) 0.01 (0.01)	4.8 (0.2) 0.25 (0.01)
HPR	(a) 11.6 (1.8) (b) 0.20 (0.03)	2.4 (0.8) 0.04 (0.02)	4.8 (3.0) 0.08 (0.03)	1.0 (1.0) 0.02 (0.02)	16.7 (2.8) 0.28 (0.05)
HPR + phosphite	(a) 11.5 (1.0) (b) 0.20 (0.03)	2.2 (0.3) 0.04 (0.01)	4.2 (1.2) 0.07 (0.02)	1.1 (1.0) 0.02 (0.02)	15.1 (2.0) 0.26 (0.03)

of which was present in the phosphocreatine reference capillary tube. Peaks appearing in the regions A, B and C indicate that small amounts (10% of total KOH-soluble P) of polyPs and nucleoside di- and triphosphates have been extracted. The doublet in region B may arise from nucleoside di- or triphosphate, although spiking experiments showed that it was not due to ATP. The ratio of peak areas in regions A and C indicates that the mean chain length of polyP in this fraction was similar to that in the PCA extract.

The spectra of KOH extracts of LPR-grown mycelia were very similar to those obtained from HPR-grown mycelia, the only difference being that the intensities of peaks in regions A, B and C were somewhat lower in spectra of LPR-grown mycelia.

Addition of phosphite to the growth medium produced

no significant changes in the spectra of the KOH extracts of either HPR- or LPR-grown mycelia.

^{32}P studies

In no case did phosphite treatment significantly affect the amount of phosphate taken up at the end of 7 d (see Table 1). The levels of phosphorus in cold PCA-soluble, lipid, KOH-soluble and hot PCA-soluble extracts are also shown in Table 1. The values for the hot-PCA soluble phosphorus should be treated with caution as these varied considerably between experiments and between duplicates within the same experiment. This variation may have been due either to the low amount of radioactivity remaining at this stage of the experiment or to incomplete extraction of KOH-soluble material. The

Fig. 5. ^{31}P NMR spectrum of live *P. palmivora* mycelium grown in HPR medium and suspended in MES buffer.

amount of hot-PCA soluble material always represented less than 10% of total mycelial phosphorus.

Treatment with phosphite under high-phosphate conditions produced no significant changes in mycelial mass or in the amount of P in any fraction. On the other hand, when mycelia were grown in LPR medium, phosphite treatment resulted in substantial changes to total phosphorus per mg dry weight and to the proportion of phosphorus in cold-PCA-soluble and lipid fractions.

The changes observed were as follows: total mycelial P (mg mycelial dry wt) $^{-1}$ increased by $80 \pm 20\%$ (mean $\pm$ SD); cold PCA-soluble P (mg mycelial dry wt) $^{-1}$ increased by $170 \pm 40\%$; lipid content decreased from 170 ± 30 to 80 ± 20 mg (mg mycelial dry wt) $^{-1}$, a decrease of $53 \pm 6\%$; lipid P (mg mycelial dry wt) $^{-1}$ increased by an average of 40%; lipid P (mg lipid) $^{-1}$ increased from 0.20 ± 0.09 to 0.58 ± 0.22 mmol, an increase of $200 \pm 68\%$.

The proportion of total mycelial P in the various fractions changed as follows: phosphorus present as PCA-soluble material increased from 0.43 to 0.60 of total P, an increase of $43 \pm 13\%$; the fraction existing as lipid P showed a very slight change from 0.23 to 0.19 (a decrease of $20 \pm 13\%$); the fraction existing as KOH-soluble P (mainly nucleic acid degradation products) decreased from 0.28 to 0.17 (a decrease of $38 \pm 13\%$).

Because no long-chain polyP was detected in *P. palmivora* the possibility of extensive polyP hydrolysis during acid extraction was investigated. A ^{31}P NMR spectrum of live HPR-grown mycelium, washed and suspended in MES buffer (pH 6.2), was obtained in order to compare the signals present with those in the various

extracts. The relative areas of terminal and non-terminal polyphosphate signals (Fig. 5) were similar to those observed in spectra of PCA extracts acquired under similar conditions. This indicated that mobile polyP in the mycelium had a mean chain length similar to that in the PCA extract. Moreover, the spectrum of water-soluble material obtained after partitioning the material removed by Bligh & Dyer (1959) extraction was similar to that of the acid extract; identical cold PCA treatment of synthetic polyP only reduced the mean chain length from 18.7 to 18.0 and the spectrum of the PCA extract of a mixture of synthetic polyP and mycelium did not show any significant breakdown of the synthetic material.

Discussion

NMR spectra and ^{32}P studies showed very marked changes in the distribution of phosphorus in *P. palmivora* treated with phosphite. One of these changes – the decrease in average polyP chain length – was evident both under conditions where phosphite inhibited growth (LPR medium) and where it did not (HPR medium).

The NMR spectra of PCA extracts showed that *P. palmivora* mycelium contains large amounts of short-chain polyP, making up between 60% (LPR) and 80% (HPR) of acid-extractable phosphorus. The distribution of ^{32}P indicated that almost all (>90%) mycelial phosphorus was accounted for in the PCA-soluble (representing 43–80% of total P), lipid and KOH-soluble fractions. This evidence, together with the lack of signals corresponding to long-chain polyP in the KOH extract,

suggested that short-chain polyP represents between 30 and 50% of total phosphorus and at least 90% (if not all) of mycelial polyP. This is unusual since most organisms which contain significant quantities of polyP accumulate it as long-chain polyP (>20 residues), short-chain polyP (<10 residues) being either absent or present in relatively small amounts (Wood *et al.*, 1986). In those cases where short-chain polyP is present, it is almost always accompanied by at least some long-chain polyP [*Tetrahymena* sp. (DesLauriers *et al.*, 1982) is one exception]. These observations are in accord with the hypothesis (Langen *et al.*, 1962; Wood & Clark, 1988) that short-chain polyP is formed from long-chain polymer. However, there is no evidence of any long-chain precursors in *P. palmivora*.

Consistent with this result, Chilvers *et al.* (1985) found no evidence of polyP granules in *Phytophthora cinnamomi* or any of the other oomycetes examined. These granules are generally (Glonek, 1971; Jacobson *et al.*, 1982) only associated with very long-chain polyP. One exception is *Tetrahymena* sp., in which the granules are made up of pyrophosphate and tripolyP (Rosenberg, 1966; DesLauriers *et al.*, 1982).

Dietrich (1976) reported the absence of polyPs in the oomycetes *Phytophthora infestans*, *Pythium* sp. and *Achlya pseudoradiosa*. This apparent conflict with our results can be resolved in a number of ways. Dietrich's method of extraction involved precipitation of polyP with Cetavlon, a reagent which precipitates large polyanionic species, or with ethanol. Small polyP (<10 residues) might not precipitate under these conditions; certainly tripolyP does not precipitate on addition of ethanol. Perhaps differences in growth conditions and media prevented formation of any polyP. It is well known (Cassone *et al.*, 1983; Guerrini *et al.*, 1980; Nicolay *et al.*, 1983; Kjeldstad & Johnsson, 1987) that polyP levels in bacteria and yeasts differ markedly with growth conditions. Finally it is possible that not all species of *Phytophthora* produce polyP.

Traditionally, polyP extraction has resulted in two fractions of polyP which are generally referred to as acid-soluble and acid-insoluble polyP. The acid used is usually perchloric (Harold, 1963; Navon *et al.*, 1979), trichloroacetic (Langen & Liss, 1958; Torriani *et al.*, 1985; Wood *et al.*, 1986) or formic (Ludwig *et al.*, 1977; Guerrini *et al.*, 1980). Acid-insoluble polyP has been extracted by a number of methods, many of which involve production of further fractions. For example, Langen & Liss (1958) followed the initial cold acid extraction with a concentrated salt extraction and a number of alkali treatments. Recently, as high-salt, hypochlorite and alkali extractions have been shown to result in some hydrolysis of long-chain polyP, extraction of acid-insoluble polyP by dilute EDTA/LiOH solution

combined with chloroform/phenol has been advocated (Wood *et al.*, 1986).

Preliminary results in this study showed that a large proportion of P was removed by PCA and lipid extraction. The extraction procedure chosen was therefore similar to that used by Goodman *et al.* (1969), where lipids were removed before one dilute alkali extraction. In the ^{32}P studies a final hot PCA extraction was performed to ensure complete extraction of any remaining phosphorus as phosphate.

It is generally accepted that acid-soluble polyP has a mean chain length of up to 20 residues (Kulaev & Vagabov, 1983; Wood *et al.*, 1986). Reports suggest that the mean chain length of acid-soluble polyP tends to vary within this limit depending on the particular organism, growth conditions and method of determination (Harold, 1966; Guerrini *et al.*, 1980; Torriani *et al.*, 1985). According to Langen & Liss (1958), acid-soluble polyP in brewer's yeast has a mean chain length between 3 and 7; Navon *et al.* (1979) calculated a mean chain length of 12–13 (not including pyrophosphate) for PCA extracts of *Saccharomyces cerevisiae*; Guerrini *et al.* (1980) separated polyphosphates of length 2–8 from acid extracts of *S. cerevisiae* but did not give the mean chain length of the total acid extract. Harold (1960) reported a mean chain length of 20, with polyP of less than seven residues being practically absent, in PCA extracts of *Neurospora crassa*. On the other hand, acid-soluble polyP in *Tetrahymena* sp. consists mainly of pyrophosphate and tripolyP (Rosenberg, 1966; DesLauriers *et al.*, 1982). Acid-insoluble polyP has much longer chain lengths, ranging from 17 to more than 1000 (Torriani *et al.*, 1985). The polyP in *P. palmivora* differs in chain length from that found in other species of fungi.

It is unclear whether the small amount of short-chain polyP appearing in the spectra of all KOH extracts in this study arises from incomplete PCA extraction, extensive base-catalysed hydrolysis of larger polyP, or authentic short polyP which is closely associated with protein or nucleic acid. The second possibility is unlikely since polyP is normally stable under basic conditions. Divalent metal cations can catalyse hydrolysis under basic conditions but it is unlikely that such species are present in significant concentrations after PCA extraction. Moreover, Wood *et al.* (1986) found that while Langen's combination of high-salt and alkaline extraction procedures *did* result in some hydrolysis of long-chain polyP (the mean chain length was approximately halved from 500 to 250 residues), most of the degradation was due to the high-salt treatment rather than the alkali.

A number of possible metabolic roles for polyP have been outlined in reviews by Kulaev & Vagabov (1983) and Wood & Clark (1988). Some of the most frequently suggested functions are as a phosphate storage pool that

may also have some osmoregulatory function, as a phosphagen and as a source of phosphate for glucose phosphorylation. However, most hypotheses have been based on the behaviour of long-chain polyP and may not be applicable to the material found in *P. palmivora*.

It remains to be seen whether the changes in polyP observed on phosphite treatment are a primary result of, or incidental to, the toxic effect of the fungicide. The fact that under high-phosphate conditions, phosphite perturbs polyP synthesis or utilization, even though no growth retardation is observed, could mean either (a) the polyphosphate changes have no connection with the toxic effect of phosphite, or (b) the presence of ample external phosphate enables the organism to bypass whatever metabolic blocks are causing the abnormal polyP distribution. The unidentified peaks in the polyP ester region of the NMR spectra of PCA-soluble HPR material may be associated with such changes.

Other major changes in phosphorylated metabolites were only observed when phosphite treatment retarded fungal growth. They included a decrease in nucleoside triphosphates and NDPG; a decrease in the amount of KOH-soluble P (mainly nucleic acid P) per mg mycelial dry weight; a very large increase in the amount of phospholipid per mg total lipid and increases in the amounts of polyP and phospholipid per mg mycelial dry weight.

These last changes would be consistent with substantial reductions in the total mass of non-phosphorylated material. The observed decrease in lipid mass per mg mycelium and the concomitant increase in the proportion of phospholipid suggest that the amount of plasma membrane may have increased at the expense of triacylglyceride storage. Fosetyl-Al treatment has already been found (Dercks & Buchenauer, 1987) to cause changes in the composition of neutral lipid and phospholipid fractions in *Phytophthora capsici* and *P. cactorum*. Morphological changes observed in the hyphae – distortion of hyphae and swelling of hyphal tips – are consistent with an increase in the amount of plasma membrane.

Although phosphonolipids such as CAEP have been reported to make up a considerable percentage of total phospholipid in *Phytophthora* (Bostock & Creamer, 1986) and *Pythium* species (Wassef & Hendrix, 1977), our NMR spectra of lipid extracts of *P. palmivora* indicated that if any phosphonolipid was present, it accounted for less than 1% of total phospholipid. The presence of small peaks in the C–P region of the KOH extract spectrum suggested that while some phosphonate or phosphonolipid was present, it was bound to insoluble material such as protein or polysaccharide. Similar compounds have been reported by Quin (1965) in the marine invertebrate *Metridium dianthus* and by Rosenberg (1964) in *Tetra-*

hymena. However, in these organisms substantial amounts of free phosphonolipid and phosphonate were also present.

This research was supported by grants from the Australian Special Rural Research Council and Albright & Wilson (Australia) Ltd to B. R. Grant.

References

- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- BOSTOCK, R. M. & CREAMER, J. R. (1986). Characterization and biological activity of *Phytophthora infestans* phospholipids in the hypersensitive response of potato tuber. *Physiological and Molecular Plant Pathology* **28**, 215–225.
- BOWER, L. A. & COFFEY, M. D. (1985). Development of laboratory tolerance to phosphorous acid, Fosetyl-Al and Metalaxyl in *Phytophthora capsici*. *Canadian Journal of Plant Pathology* **7**, 1–6.
- CASSONE, A., CARPINELLI, G., ANGIOLELLA, L., MADDALUNO, G. & PODO, F. (1983). ³¹P nuclear magnetic resonance study of growth and dimorphic transition in *Candida albicans*. *Journal of General Microbiology* **129**, 1569–1575.
- CHILVERS, G. A., LAPEYRIE, F. F. & DOUGLASS, P. A. (1985). A contrast between oomycetes and other taxa of mycelial fungi in regard to metachromatic granule formation. *New Phytologist* **99**, 203–210.
- COFFEY, M. D. & BOWER, L. A. (1984). *In vitro* variability of eight *Phytophthora* species in response to phosphorous acid. *Phytopathology* **74**, 738–741.
- COFFEY, M. D. & JOSEPH, M. C. (1985). Effects of phosphorous acid and Fosetyl-Al in the life cycle of *Phytophthora cinnamomi* and *P. citricola*. *Phytopathology* **75**, 1042–1045.
- COHEN, Y. & COFFEY, M. D. (1986). Systemic fungicides and the control of Oomycetes. *Annual Review of Phytopathology* **24**, 311–338.
- DERCKX, W. & BUCHENAUER, H. (1987). Comparative studies on the mode of action of aluminium ethyl phosphite in four *Phytophthora* species. *Crop Protection* **6**, 82–89.
- DESLAURIERS, R., EKIEL, I., BYRD, R. A., JARRELL, H. C. & SMITH, I. C. P. (1982). A ³¹P-NMR study of structural and functional aspects of phosphate and phosphonate distribution in *Tetrahymena*. *Biochimica et Biophysica Acta* **720**, 329–337.
- DIETRICH, S. M. C. (1976). Presence of polyphosphate of low molecular weight in *Zygomycetes*. *Journal of Bacteriology* **127**, 1408–1413.
- DITTMER, J. C. & LESTER, R. L. (1964). A simple specific spray for the detection of phospholipids on thin layer chromatograms. *Journal of Lipid Research* **5**, 126–127.
- DOLAN, T. E. & COFFEY, M. D. (1988). Correlative *in vitro* and *in vivo* behaviour of mutant strains of *Phytophthora palmivora* expressing different resistances to phosphorous acid and fosetyl-Al. *Phytopathology* **78**, 974–978.
- FENN, M. E. & COFFEY, M. D. (1984). Studies on the *in vitro* and *in vivo* antifungal activity of Fosetyl-Al and phosphorous acid. *Phytopathology* **74**, 606–611.
- GLONEK, T., LUNDE, M., MUDGETT, M. & MYERS, T. C. (1971). Studies of biological polyphosphate through the use of phosphorus-31 nuclear magnetic resonance. *Archives of Biochemistry and Biophysics* **142**, 508–513.
- GOODMAN, E. M., SAUER, H. W., SAUER, L. & RUSCH, H. P. (1969). Polyphosphate and other phosphorus compounds during growth and differentiation of *Physarum polycephalum*. *Canadian Journal of Microbiology* **15**, 1325–1331.
- GRANT, B. R., GRIFFITH, J. M., IRVING, H. R. & RADD, M. (1984). An improved method for the synchronous production of zoospores from *Phytophthora palmivora*. *Experimental Mycology* **8**, 382–385.

- GUERRINI, A. M., NAZZARENA, B. & DONINI, P. (1980). Chromatographic separation and identification of short-chain acid-soluble polyphosphates from *Saccharomyces cerevisiae*. *Journal of Chromatography* **189**, 440–444.
- GUEST, D. I. (1984). Modification of defence responses in tobacco and capsicum following treatment with fosetyl-Al. *Physiological Plant Pathology* **24**, 125–134.
- HAROLD, F. M. (1960). Accumulation of inorganic polyphosphate in mutants of *Neurospora crassa*. *Biochimica et Biophysica Acta* **45**, 172–188.
- HAROLD, F. M. (1963). Accumulation of inorganic polyphosphates in *Aerobacter aerogenes*. *Journal of Bacteriology* **86**, 216–221.
- HAROLD, F. M. (1966). Inorganic polyphosphates in biology: structure, metabolism, and function. *Bacteriological Reviews* **30**, 772–794.
- HENDRIX, J. W. & ROUSER, G. (1976). Polar lipids of *Phytophthora parasitica* var. *nicotianae*, in comparison with those of selected other fungi. *Mycologia* **68**, 354–361.
- JACOBSON, L., HALMANN, M. & YARIV, J. (1982). The molecular composition of the volutin granule in yeast. *Biochemical Journal* **201**, 473–479.
- KJELDSTAD, B. & JOHNSON, A. (1987). A ^{31}P -NMR study of *Propionibacterium acnes*, including effects caused by near-ultraviolet irradiation. *Biochimica et Biophysica Acta* **927**, 184–189.
- KULAEV, I. S. & VAGABOV, V. M. (1983). Polyphosphate metabolism in microorganisms. *Advances in Microbial Physiology* **24**, 83–171.
- LANGEN, P. & LISS, E. (1958). Über Bildung und Umsatz der Polyphosphate der Hefe. *Biochemische Zeitschrift* **330**, 455–466.
- LANGEN, P., LISS, E. & LOHMAN, K. (1962). Art, Bildung und Umsatz der Polyphosphate der Hefe. *Colloques Internationaux du Centre National de la Recherche Scientifique* **106**, 603–612.
- LUDWIG, J. R., II, OLIVER, S. G. & McLAUGHLIN, C. S. (1977). The effect of aminoacids on growth and phosphate metabolism in a prototrophic yeast strain. *Biochemical and Biophysical Research Communications* **79**, 16–23.
- NAVON, G., SHULMAN, R. G., YAMANE, T., ECCLESHALL, T. R., LAM, K.-B., BARONOFKY, J. J. & MARMUR, J. (1979). Phosphorus-31 nuclear magnetic resonance studies of wild-type and glycolytic pathway mutants of *Saccharomyces cerevisiae*. *Biochemistry* **18**, 4487–4499.
- NICOLAY, K., SCHEFFERS, W. A., BRUINENBERG, P. M. & KAPTEIN, R. (1983). In vivo ^{31}P NMR studies on the role of the vacuole in phosphate metabolism in yeasts. *Archives of Microbiology* **134**, 270–275.
- QUIN, L. D. (1965). The presence of compounds with a carbon-phosphorus bond in some marine invertebrates. *Biochemistry* **4**, 324–330.
- RIBEIRO, O. K., ERWIN, D. C. & ZENTMEYER, G. A. (1965). An improved synthetic medium for oospore production and germination of several *Phytophthora* species. *Mycologia* **67**, 1012–1019.
- ROSENBERG, H. (1964). Distribution and fate of 2-aminoethylphosphonic acid in *Tetrahymena*. *Nature, London* **203**, 299–300.
- ROSENBERG, H. (1966). The isolation and identification of 'volutin' granules from *Tetrahymena*. *Experimental Cell Research* **41**, 397–410.
- SCHWINN, F. J. & URECH, P. A. (1986). Progress in the chemical control of diseases caused by Oomycetes. In *Fungicide Chemistry: Advances and Practical Applications* (ACS Symposium no. 304), pp. 89–106. Edited by M. B. Green & D. A. Spiller. Washington, DC: American Chemical Society.
- STILLWAY, L. W. & HARMON, S. J. (1980). A procedure for detecting phosphonolipids on thin layer chromatograms. *Journal of Lipid Research* **21**, 1141–1143.
- TORRIANI, A., RAO, N. N. & ROBERTS, M. F. (1985). Amount and chain length of polyphosphates in *Escherichia coli* depend on cell growth conditions. *Journal of Bacteriology* **162**, 242–247.
- WASSEF, M. K. & HENDRIX, J. W. (1974). Lipid content of cured tobacco. *Canadian Journal of Botany* **52**, 1123–1126.
- WASSEF, M. K. & HENDRIX, J. W. (1977). Ceramide aminoethylphosphonate in the fungus *Pythium prolatum*. *Biochimica et Biophysica Acta* **486**, 172–178.
- WOOD, H. G. & CLARK, J. E. (1988). Biological aspects of inorganic polyphosphates. *Annual Review of Biochemistry* **57**, 235–260.
- WOOD, H. G., CLARK, J. E. & BEEGEN, H. (1986). Isolation of intact chains of polyphosphate from '*Propionibacterium shermanii*' grown on glucose or lactate. *Journal of Bacteriology* **168**, 1212–1219.