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Acid Phosphatase and Esterase Activity in Orchid Mycorrhiza

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Summary. A cytochemical study was made to examine the possibility that acid phosphatase may be specifically involved in the digestion of endophytic hyphae in orchid mycorrhiza. Esterase activity was studied for comparison. Frozen sections of unfixed or glutaraldehyde-fixed protocorms of *Dactylorhiza purpurella* infected by *Thanatephorus cucumeris* (*Rhizoctonia solani*) were reacted for acid naphthol AS BI phosphatase, acid β-glycerophosphatase or naphthol AS D esterase.

A marked increase in particulate acid naphthol AS BI phosphatase activity was observed during infection of host, central, parenchyma cells shortly before hyphae lysed; a diffuse reaction of high activity was localised on lysed fungus. Acid β-glycerophosphatase was present at particulate sites only in fungal cytoplasm and as a diffuse reaction on lysed fungus.

Naphthol AS D esterase showed highest activity at hyphal apices. Esterase seems to be associated with growth and differentiation of hyphae in orchid cells, rather than lysis of the fungus.

Introduction

Fungi which form mycorrhizal associations with orchids are digested in the central parenchyma cells (see Harley, 1969). It is not known which partner in the mycorrhiza synthesises the enzymes which lyse intracellular hyphae. Since achlorophyllous orchids gain nutrients from the intimate fungal association (Hadley and Williamson, 1971), it has been assumed that digestive enzymes are produced by the host (Gallaud, 1905; Bernard, 1909; Burges, 1939; Burgeff, 1959). This assumption may be unfounded, for many fungi undergo lysis following the release of self-synthesised hydrolases (autolysis) during starvation (Trinci and Righelato, 1970) or during maturation of fruit bodies (Iten and Matile, 1970). In the cytoplasm of orchid parenchyma cells hyphae may experience nutritional stress and autolyse. It may be, therefore, that either the host or the endophyte (or both) synthesise hydrolases prior to lysis of the fungus.

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In this paper the distribution of two acid phosphatase activities in orchid cells is described during successive stages of symbiotic infection and fungal lysis. The distribution of naphthol AS D esterase activity is described for comparison.

Materials and Methods

Plant Materials

Axenic Symbiosis Cultures. Seeds of *Dactylorhiza purpurella* (T. and T. A. Steph.). Soó were surface sterilised and sown in Pfeffer agar (Williamson and Hadley, 1970). The medium was supplemented with 2% microgranular cellulose powder (Chromedia CC41) as carbon source (Hadley, 1969). Orchid cultures were incubated at 24° C in darkness until germination occurred.

Thanatephorus cucumeris (Frank) Donk. (*Rhizoctonia solani* Kühn), Isolate 82 is symbiotic with protocorms of *D. purpurella* (Williamson and Hadley, 1970). The fungus was maintained on potato dextrose agar, and subcultured to Pfeffer agar before inoculation of orchid cultures. Mycorrhizal protocorms were sampled at intervals up to 37 days following inoculation.

Preparation of Tissues

Protocorms were either (a) fixed in 2.5% glutaraldehyde in 0.1 M cacodylate/HCl buffer at pH 7.1 for 2 h at 4° C, and washed overnight in buffer (Sebatini *et al.*, 1963); or (b) used unfixed. Protocorms were briefly embedded at 26° C in a gelatin support medium containing glycerine (Knox and Heslop-Harrison, 1970) for freeze-sectioning by the method of Gahan *et al.* (1967).

Enzyme Methods

Sections were incubated for 2, 5, 10, 20, 30, 45 or 60 min at 37° C in the described reaction media.

1. Acid Naphthol AS BI Phosphatase. The azo-dye method of Burstone (1958) was employed. Naphthol AS BI phosphate (0.32 mM) was used as substrate in the presence of Fast Red Violet LB coupling salt at 1.4 mg/ml in 0.2 M acetate buffer at pH 5.1. Control sections were incubated in either the medium with the substrate omitted, or the full reaction medium plus 10 mM sodium molybdate.

2. Acid β-glycerophophatase. A modified Gomori reaction medium (Holt, 1959) was used which contained 14 mM sodium β-glycerophosphate in 0.05 M actate buffer at pH 5.1. Control sections were incubated in either the medium with substrate omitted, or the complete medium plus 10 mM sodium fluoride. The 1% acetic rinse (Gomori, 1952) was employed for precisely 10 seconds. Lead phosphate end product was converted to visible lead sulphide by the method of Bitensky (1963).

3. Naphthol AS D Esterase. The method of Burstone (1957) was employed using 0.31 mM naphthol AS D acetate as substrate with Fast Red Violet LB Salt as coupling agent at 0.6 mg/ml in 0.1 M Tris/HCl buffer at pH 7.1. Control sections were incubated in medium lacking substrate.

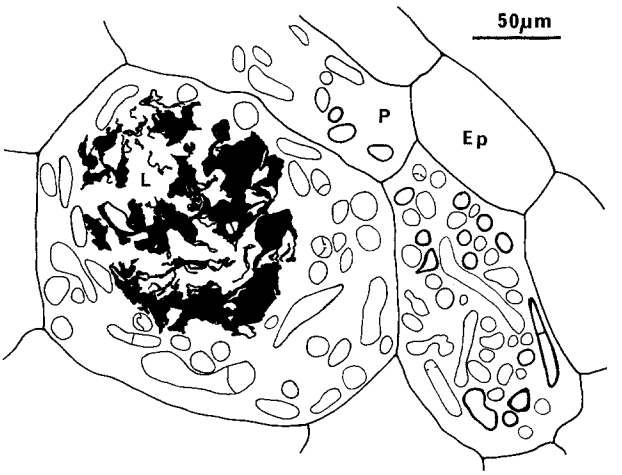


Fig. 1. Cells of protocorm of *D. purpurella* infected by *T. cucumeris*. Diagram from photographs of epoxy resin sections (0.15 µm). *Ep* epidermis, uninfected; *P* peripheral parenchyma infected by thick or thin-walled hyphae; *L* lysed fungus surrounded by hyphae of second infection in central parenchyma

Cytology

Protocorms were fixed in glutaraldehyde (as above), sliced at 100 µm on a Sorvall TC-2 Tissue slicer, post-fixed in 1% osmic acid, dehydrated through ethanol and embedded in Spurr's epoxy resin (Spurr, 1969). Sections of 0.15 µm were taken on a Reichert Ultramicrotome and stained with toluidine blue for light microscopy.

Results

Morphological Observations

A marked difference in the infection pattern was observed between peripheral and central parenchyma cells (Fig. 1). In the former, hyphae were regular in diameter (7–11 µm) and maintained at relatively low density in each cell. Some hyphae synthesised thick walls (Fig. 1). Pelotons in peripheral parenchyma began to lyse about 26 days after inoculation. Thick walled hyphae did not collapse on lysis. The central parenchyma cells were infected by thin-walled hyphae of more variable diameter (7–19 µm). In some cells the cytoplasm of a few hyphae stained strongly with toluidine blue.

The following cytological features were seen in sections of cells, which from previous studies on living slide cultures (Williamson and Hadley, 1970) clearly show a host cell being infected repeatedly.

Stage 1: Few turgid hyphae per cell (primary infection).

Table 1. Minimum incubation times required to yield coloured end product in glutaraldehyde fixed central parenchymal cells 15 days after inoculation

	Acid β-glycero-phosphatase		Acid naphthol AS BI phosphatase		Naphthol AS D esterase	
	orchid	hyphae	orchid	hyphae	orchid	hyphae
Primary infection						
1.	>60	10–20	45	>60	10	5–10
2.	45–60[a]	10–20	10	10	10	10
3. lysis	45[a]	2	20	2	>60	>60
Secondary infection						
4.	>60	10–20	45	>60	10	10
5.	45–60[a]	10–20	10	10	10	10
6. lysis	45[a]	2	20	2	>60	>60

[a] Diffuse reaction attributed to cover incubation of cells with high activity in hyphae.

Stage 2: Many turgid hyphae occluding host cell.

Stage 3: Only collapsed fungal walls present (lysis).

Stage 4: Collapsed fungal walls surrounded by few turgid hyphae (secondary infection).

Stage 5: Collapsed fungal walls and turgid hyphae occluding host cell.

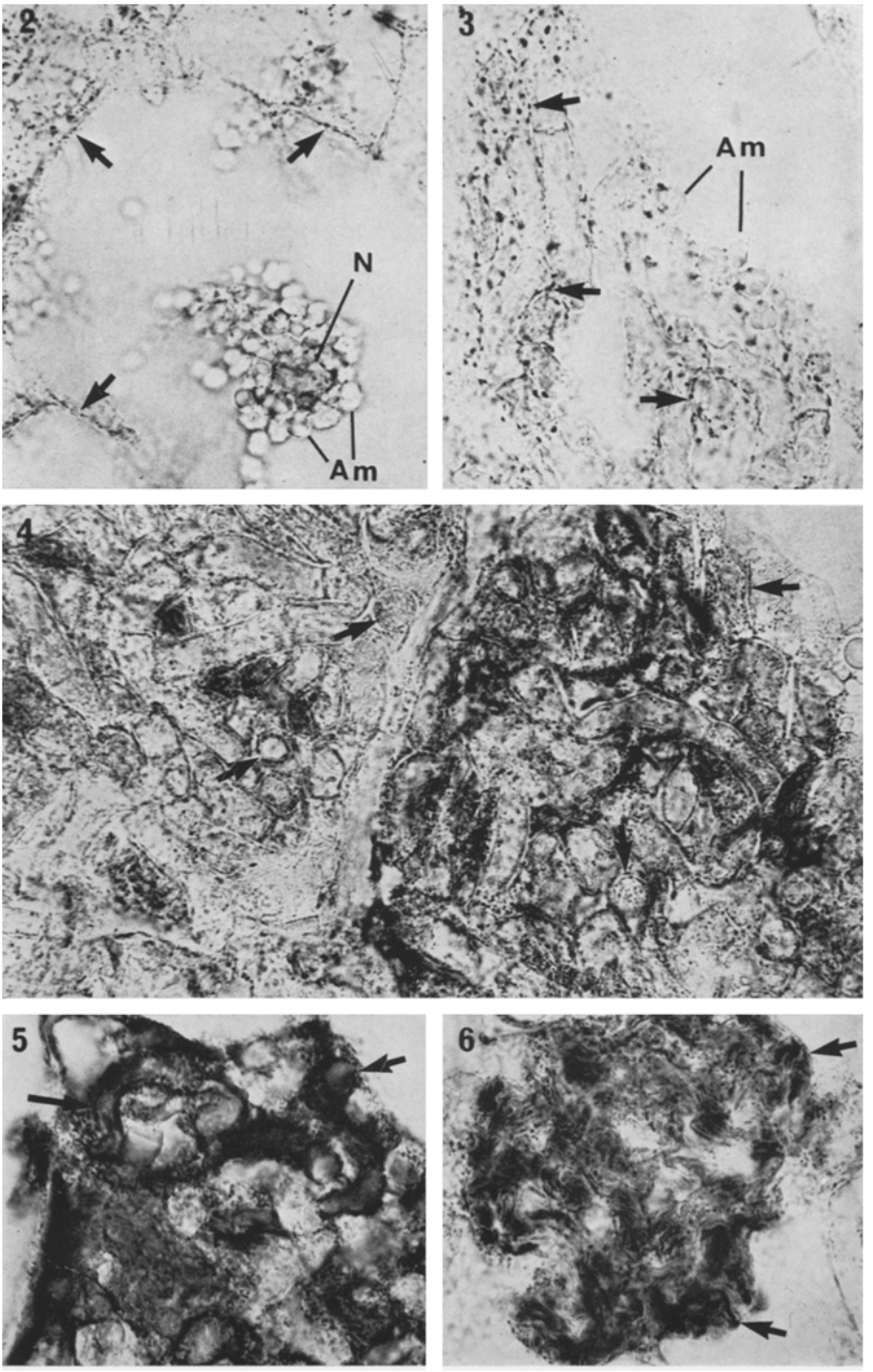
Stage 6: Two groups of collapsed fungal walls, separate or stratified (secondary lysis).

Lysed pelotons were present in some central parenchyma cells within 4 days of inoculation. Pelotons lysed in neighbouring host cells asynchronously. Some hyphal compartments within a peloton do not appear to lyse immediately.

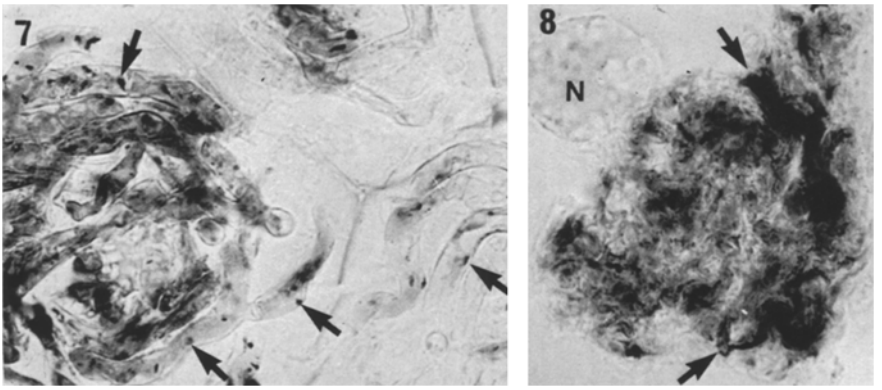
Localisation of Enzymes

The incubation of tissues giving a particulate reaction until diffusion occurs has been used as one illustration of the "latency" of sequestered lysosomal enzymes (Gahan, 1965). In these experiments it was hypo-

Figs. 2—6. Distribution of acid naphthol AS BI phosphatase in central parenchyma cells at various stages of infection. Fig. 2. Particulate reaction in uninfected cells close to plasmalemma. Glutaraldehyde-fixed (incubated 45 min). Fig. 3. Primary infection, stage 1. Particulate reaction in host membrane surrounding hyphae. Glutaraldehyde-fixed (incubated 20 min). Fig. 4. Hyphae occlude two host cells, Stage 2. Particulate reaction now visible in hyphae. Unfixed, frozen (incubated 45 min). Fig. 5. Diffuse reaction in some morphologically intact hyphae. Glutaraldehyde-fixed (incubated 30 min). Fig. 6. Diffuse reaction localised on lysed fungus. Glutaraldehyde-fixed (incubated 20 min). *Am* amyloplasts, *N* host nucleus. End product indicated by arrows (↙). ×640



Figs. 2—6

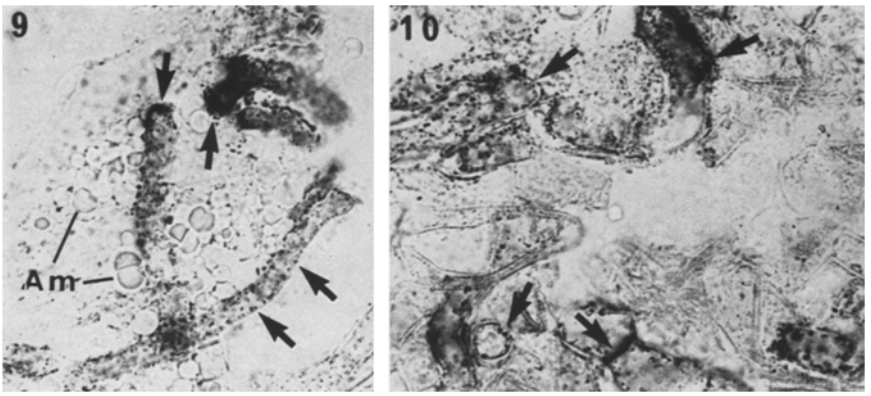


Figs. 7 and 8. Distribution of acid β-glycerophosphatase in glutaraldehyde-fixed cells. Fig. 7. Fluoride sensitive particulate reaction becoming diffuse in morphologically intact hyphae (incubated 20 min). Fig. 8. Fluoride sensitive diffuse reaction on lysed hyphae (incubated 20 min). *N* host nucleus. End product indicated by arrows (↙). ×640

thesised that a diffuse reaction following minimal incubation times localized enzyme released during fungal lysis, rather than by over-incubation of membrane-bound enzyme.

An indication of the relative enzyme activities observed in central parenchyma cells during successive stages of infection is shown in Table 1. The sites producing enzyme end product after brief incubation periods are assumed to localise the highest enzyme activity. When no end product was produced during a 60 min incubation, activity for such enzymes was considered to be neglible or absent.

Acid Naphthol AS BI Phosphatase. The distribution of acid naphthol AS BI phosphatase was the same in fresh frozen and glutaraldehyde fixed tissues. Uninfected parenchyma cells showed activity within 45 min incubation (Fig. 2) at particulate sites close to the plasmalemma. Activity in central parenchyma cells remained low during early infection (stages 1 and 4), and was localised at particulate sites near a host membrane surrounding each intracellular hypha (Fig. 3). The cytoplasm of hyphae showed no activity at these stages. When hyphae completely occluded the host cell (stages 2 and 5) activity in host cytoplasm was high and particulate sites were then present in fungal cytoplasm. The overall activity in two adjacent cells prior to lysis may be different (Fig. 4). A diffuse reaction of high activity was observed in some morphologically intact hyphae at stages 2 and 5 (Fig. 5). Activity was absent in some hyphae which appeared highly refractile under



Figs. 9 and 10. Distribution of naphthol AS D esterase in unfixed, frozen sections of central parenchyma cells. Fig. 9. Particulate reaction in invading hyphae and associated host membranes; diffuse reaction at hyphal apices. Fig. 10. Activity at hyphal septa and some isolated compartments in established peloton. *Am* amyloplasts. Activity indicated by arrows (↙). ×640

bright-field microscopy. These hyphae remained intact when most hyphae in the cell had lysed. Lysed pelotons (stages 3 and 6) invariably showed high activity giving a largely diffuse reaction (Fig. 6). Omission of the substrate, or the addition of sodium molybdate inhibited all activity.

In peripheral parenchyma cells, acid naphthol AS BI phosphatase was present at particulate sites in thin-walled hyphae; though absent in thick-walled hyphae. A diffuse reaction was observed on lysed hyphae 3–5 weeks after inoculation.

Acid β-glycerophosphatase. The distribution of end product differed in unfixed, frozen and glutaraldehyde-fixed sections. However, in neither treatment was activity attributed to the host cytoplasm. Glutaraldehyde-fixed free-living hyphae, and all morphologically intact hyphae in orchid cells showed activity within 10 to 20 min incubation at particulate or vesicular sites in fungal cytoplasm (Fig. 7). With longer incubation times these reactions became diffuse; the adjacent host cytoplasm reacted and nuclear staining occurred. Lysed pelotons (stages 3 and 6) showed a diffuse reaction of very high activity (Fig. 8). Not all lysed hyphae showed acid β-glycerophosphatase activity. All activity in glutaraldehyde fixed sections was completely inhibited by omission of substrate, or the presence of 10 mM sodium fluoride.

In unfixed, frozen sections, end product was present at numerous particulate sites in hyphae within 2 min. This "reaction" was not abolished by the controls employed.

Naphthol AS D Esterase. Uninfected orchid cells showed esterase activity at particulate sites near the plasmalemma. In central and peripheral parenchyma cells the highest esterase activity was present in the early stages of infection (stages 1 and 4) associated with the membrane surrounding the apices of invading hyphae (Fig. 9). Fungal cytoplasm at this site gave a strong particulate reaction and a diffuse reaction in the hyphal wall. During stages 2 and 5 end product was largely associated with fungal septa and dolipore apparatus in isolated compartments of each peloton (Fig. 10). No esterase activity was detected in lysed fungus. Controls omitting substrate were negative. In established pelotons in peripheral parenchyma cells a particulate reaction was present only in thin-walled hyphae.

Discussion

Acid phosphatase was localized as a diffuse reaction on lysed fungus in orchid cells by two independent techniques. The close correlation between the known site of hyphal lysis and the highest acid phosphatase activity is interesting. Since similar reactions were obtained for unfixed, frozen and glutaraldehyde-fixed cells, the diffuse reaction is more likely to result from release of sequestered enzyme during lysis, than from repeated freezing and thawing of cells which occurs during cryostat sectioning (see, Pearse, 1968).

It is known that the lead-salt method and the azo-dye technique localise acid phosphatase with characteristic substrate specificities (Rosenbaum and Rolon, 1962; Meany *et al.*, 1967). Therefore, it was not surprising that the distribution of acid β-glycerophosphatase differed from acid naphthol AS BI phosphatase. The latter was detected at particulate sites in all orchid cells and therefore may be considered "constitutive". The increased activity observed during infection of central parenchyma cells could result from activation or synthesis of "constitutive" enzyme, or *de novo* synthesis of a different phosphatase (which also cleaves the phosphomonoester linkage in naphthol AS BI phosphate) associated with fungal digestion. Activity was present in some thin-walled hyphae in peripheral parenchyma, and hyphae in central parenchyma shortly before lysis. Does *de novo* synthesis of a fungal phosphatase occur in endophytic hyphae? Since both organisms show a particulate reaction prior to fungal lysis it was not possible to attribute the diffuse reaction following lysis to either fungus or host.

The distribution of acid β-glycerophosphatase must be interpreted with caution for the end product observed following glutaraldehyde fixation differed from that seen in unfixed, frozen sections. In the latter, end product was not completely inhibited by controls. It is possible

that glutaraldehyde selectively inhibits enzyme activity at some cytoplasmic sites in fungal hyphae, and that this phosphatase is fluoride resistant. In the absence of added substrate an endogenous source of phosphate may be localised (Sommer and Blum, 1965). However, localisations shown to be fluoride sensitive were accepted as sites of acid phosphatase activity in this study. It is surprising that acid β-glycerophosphatase was not localised in orchid cytoplasm, for this activity is widespread in higher plant tissues.

Naphthol AS D esterase was associated with early stages of infection, showing highest activity at hyphal apices. In contrast to acid naphthol AS BI phosphatase, it does not seem to be involved with fungal lysis, but rather with hyphal growth and differentiation. This supports previous evidence that naphthol AS D esterase activity is associated with cell wall changes.

Acid phosphatases may be expected to cleave phosphomonoester linkages in phospholipid membranes when released from compartmentalised sites in cells, but the precise intracellular substrate(s) are unknown. Doubts concerning the functional significance of this enzyme have lead to suggestions that acid proteases, nucleases and carbohydrases be taken as markers for plant lysosomal systems (Matile, 1969) rather than acid phosphatase hitherto used widely for this purpose.

It is suggested that large increases in phosphatase activity in either organism may result from the depletion of phosphate-ester pools, or other nutrients, in cells supporting extensive hyphal growth. Acid phosphatase could therefore be involved in phosphate recycling as the fungus lyses. This hypothesis might explain the increase in particulate acid naphthol AS BI phosphatase occurring in both host and endophyte, for presumably both organisms would experience nutrient stress.

It is unresolved whether endophytic hyphae lyse autolytically in orchid cells or are digested by enzymes secreted by the host.

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