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Hyphal Tip Growth in *Phytophthora*

Gradient Distribution and Ultrahistochemistry of Enzymes

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Abstract. Germinating cysts and isolated walls from germinating cysts incorporated ^{14}C-UDPG into wall material of which 22.5 and 15% respectively were insoluble in boiling 1 N HCl, indicating that part of the synthetase activity is located in the wall itself. A combination of Urografin and Ficoll density gradients was used to separate various intracellular fractions. A consistent separation of β-glucanase and UDPG-transferase enriched fractions was achieved. The β-glucanase fraction contained dictyosome vesicles and fragments along with some plasma membranes. The UDPG-transferase fraction was relatively rich in membranes resembling rough and smooth ER. The results suggest the two enzymes are transported to the wall by different intracellular routes, and two types of vesicle may be involved. Alkaline phosphatase, β-glucosidase and acid phosphatase were found extracellularly and their distribution in density gradients determined. The results of histochemical staining for acid phosphatase, alkaline phosphatase and polysaccharide are described and compared with the biochemical data. β-1,3-glucanase, found intra- and extracellularly, induced distorted growth of germ tubes and also removed most of the apical wall when added to the incubation medium. None of these responses were observed with cellulase. Determinations of the osmotic pressure of germinating cysts and incubation medium revealed that the turgor of germinating cysts amounts to about 1.8 at under the conditions used.

Key words: Phytophthora palmivora – UDPG-transferase – Alkaline phosphatase – Acid phosphatase – β-1,3-glucanase – Wall formation – Hyphal tip – Osmotic pressure – Enzymatic wall degradation.

The presently held concept of hyphal tip growth, as aptly summarized by Bartnicki-Garcia (1973), maintains that the hyphal apex is kept in a pliable condition by a delicate balance of wall synthesis and wall lysis whereby the driving force for wall extension is provided by the turgor of the fungal protoplast. As the newly formed wall gradually moves into a lateral position it stiffens and looses its extensibility. While it has been possible to demonstrate in several fungi the presence of wall synthesizing and/or wall lysing enzymes, there is at present no single case where the presence and functioning of all the necessary components has been established.

The components include: (1) the necessary turgor pressure, (2) formation, transport and site of activity of the lytic and synthesizing machinery, (3) source and transport of wall precursors, (4) mechanism for restriction of wall synthesis to the hyphal apex, and (5) existence of a crucial, delicate balance between lytic and synthetic activities.

Any attempt to explain the intricate mechanism of hyphal tip growth will have to take into account the special cytological features prevailing at the hyphal tip, i.e. the presence of numerous small vesicles characteristically present in many species (Girbardt, 1969; Grove and Bracker, 1970). Indirect evidence derived mostly from ultrastructural studies, suggests that these vesicles play a decisive role in wall formation by transporting wall precursors and/or the necessary enzymes to the cell periphery. Hyphal tip growth, therefore, appears to be intimately connected with the activity of the endomembrane system characteristic for eukaryotic cells (Morré et al., 1971; Morré and Mollenhauer, 1974).

In a effort to gain a better understanding of the structural and functional relationships in the hyphal tip we have used biochemical and histochemical techniques to localize relevant intra- and extracellular enzymes and substrates. The germinating cysts of *Phytophthora palmivora* were selected because the germ tube tip represents a relatively large portion of the total cell volume and this species is well character-

ized from an ultrastructural (Hemmes and Hohl, 1969, 1971; Hunsley and Burnett, 1970; Gooday, 1971; Tokunaga and Bartnicki-Garcia, 1971a, b; Hunsley, 1973; Hegnauer and Hohl, 1973; Desjardins et al., 1973) and a biochemical point of view (Bartnicki-Garcia, 1966; Wang and Bartnicki-Garcia, 1966, 1973; Tokunaga and Bartnicki-Garcia, 1971a, b). Since glucans make up 90% of the wall material (Bartnicki-Garcia, 1966) in form of cellulose and β-1,3-(1,6)-polymers, major emphasis was placed on the investigation of glucanase and glucan-synthetase. In addition several other enzymes were included as markers for specific cell fractions or because of their potential involvement in tip growth.

MATERIAL AND METHODS

Culture Methods

Phytophthora palmivora (Butler) P113 (ATCC 26286) was grown in the dark at 31°C for 4–5 days on V-8 medium (10% V-8 vegetable juice, 0.1% $CaCO_3$ and 1.5% agar in water). The cultures were illuminated for 24 h at room temperature to induce sporangia.

Cultures were also grown in liquid, modified P-1L medium (Hohl, 1975) containing (g/l in H_2O): $NaNO_3$ (3.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), KCl (0.5), $FeSO_4 \cdot 7H_2O$ (0.01), $CaCl_2 \cdot 2H_2O$ (0.1), $FeCl_3$ (0.001), $ZnCl_2$ (0.001), EDTA (0.01), thiamine · HCl (0.0005), asparagine · H_2O (1.0), sucrose (10.0) and lecithin Type IIE from egg yolk, Sigma (0.2). The medium was inoculated with sporangia and incubated in the dark at 31°C for 5 days. The filtrate (Whatman Nr.1 filter) was used for the determination of extracellular enzymes.

Preparation of Germinating Cysts

Double dist. water (8 ml per plate; pH 6; 25–28°C) was poured onto cultures containing sporangia. After 25 min the zoospores that had been released from sporangia were decanted into an Erlenmeyer flask. The suspension was diluted with Casamino acids (Difco 1.5% in double dist. H_2O) to give a concentration of 2×10^5 cells/ml. Under these condition cysts formed and began to germinate after 40–60 min at 28°C. Germinating cysts were used for organelle isolation when the length of the germ tube was twice the cyst diameter.

Isolation of Cell Walls

Germinating cysts were collected by centrifugation and homogenized in a French press (Aminco, Silver Spring) in double dist. water. The wall fragments were washed several times with water, then sonicated for 60 s (setting 30 on a Braun Sonifier) and washed again.

Preparation of the Homogenate

The suspension of germinating cysts was centrifuged (1000 × g, 10 min) and 5 ml of SF medium added (0.5 M sorbitol, 2.5% Ficoll, 0.5% BSA in 0.05 M Tris-maleate, pH 7.1). Glassbeads were added (3 ccm, 0.35 mm diam.) and the suspension homogenized in a cell mill (Bühler, Tübingen) at 4°C. Whole cysts and cell wall fragments were removed by first filtering the homogenate through a nylon cloth (pore size 63 µm) and then centrifuging (1000 × g, 3 min). The supernatant was used in the separations described in the next section.

Separation of Cell Components

a) Differential Centrifugation. Fractions were obtained by centrifuging the homogenate for 25 min (4°C) at 8000 × g, 23000 × g and 50000 × g using a Beckman J-21 centrifuge and a Beckman L2-65B ultracentrifuge.

b) Density Gradients. Two different gradients were employed: a linear Urografin (methylglucamine salt of N,N-diacetyl-3,5-diamino-2,4,6-triiodobenzoic acid; Schering AG, Berlin) and a Ficoll step gradient.

The Urografin was dissolved in 0.05 M Tris-maleate (pH 7.1) containing 0.1 M sorbitol and linear gradients of 1.05–1.24 g/ml on a cushion of 70% (w/v) sucrose were prepared. The material sedimenting between 8000 × g (25 min) and 23000 × g (25 min) was resuspended in SF medium and loaded onto the gradients.

To two lowest bands obtained after centrifugation on Urografin gradients were removed, diluted in 0.05 M Tris-maleate, pH 7.1 and loaded on Ficoll step gradients. These were prepared from 11%, 16%, 20% and 25% (w/v) Ficoll (1 ml each) in 0.05 M Tris-maleate (pH 7.1). This corresponds to densities of 1.05, 1.06, 1.07, and 1.09 g/ml respectively. All gradients were centrifuged for 2 h at 4°C and 150000 × g in a Spinco SW-50 rotor and 10–15 fractions per gradient were subsequently collected.

Protein, Polysaccharide and Enzyme Assays

Protein was determined by the method of Lowry et al. (1951), being first precipitated from Urografin gradient fractions with 10% TCA.

Polysaccharide was measured by the anthrone test (Glegg, 1956). Fractions from the Ficoll gradient were first suspended in 4.0 ml 0.05 M Tris-maleate (pH 7.1), centrifuged at 23000 × g (20 min) and the sediment resuspended in 0.4 ml buffer.

The activities of acid and alkaline phosphatase, α-mannosidase, β-glucosidase and β-galactosidase were assayed with p-nitrophenyl linked chromogenic substrates (Parish, 1975).

Malate dehydrogenase and citrate synthetase were measured according to the methods described by Boehringer Inc., Mannheim. Thiamine pyrophosphatase (TPPase) and adenosine monophosphatase (AMPase) were assayed in 0.1 M Tris-HCl containing 4 mM TPP and 5 mM $MgCl_2$ (pH 7.5) and 10 mM AMP and 10 mM $MgCl_2$ (pH 8.5) respectively. The phosphate released was measured according to Fiske and Subbarow (1925).

The $NADH_2$-ferricyanide reductase was assayed in 0.1 M Tris-HCl containing 1 mM $K_3Fe(CN)_6$, 1 mM KCN and 0.1 mM $NADH_2$. The reduction of ferricyanide was measured at 400 nm.

Exo-β-1,3-glucanase was measured according to Abd-El-Al and Phaff (1968), with the addition of 0.05% Triton X-100.

Uridine diphosphoglucose (UDPG) transferase was measured by the method of Ray et al. (1969). The reaction mixture consisted of 50–100 µl enzyme, 50 mM $MgCl_2$ and 0.26 µCi UDP-14C-glucose (250 mCi/mmol, Radiochemical Centre) in 0.05 M Tris-HCl buffer (pH 7.2). The controls contained boiled enzyme. The reaction was stopped by adding 4.0 ml of boiling 70% ethanol containing 0.1% Cellite.

The sediment was suspended in 0.5 ml 70% ethanol, transferred to filter paper (in a POPOP-toluol mixture) and radioactivity measured in a Unilux I (Nuclear Chicago) scintillation counter.

The UDPG-transferase activity of isolated cell walls was measured by suspending the material in 0.05 M Tris-HCl (pH 7.1)

and incubating as described above except that the walls were washed with water following incubation.

Electron Microscopy

Hyphae, germinated cysts and cellular fractions were fixed for 30–60 min in 2.5% cacodylate buffered glutaraldehyde at pH 7.2, sedimented, washed extensively with buffer and refixed for 1 h in 1% cacodylate buffered osmiumtetroxide at pH 7.2. The material was then washed again, dehydrated in ethanol and propylene oxide and embedded in Epon. Ultrathin sections were stained in 1% aqueous uranyl acetate and lead citrate and examined in a Hitachi HU-11E electron microscope.

Histochemistry

Acid and alkaline phosphatase activity were detected using the lead phosphate reaction as described by Ericsson and Trump (1964) and by Hugon and Borgers (1966) respectively.

Staining ultrathin sections for polysaccharides was performed according to the periodate-thiosemicarbazide-silver proteinate-reaction described by Thiéry (1967). Controls were obtained by omitting the coupling reagent, thiosemicarbazide, from the procedure.

Enzymatic Wall Degradation

Germlings were prefixed in 2.5% buffered glutaraldehyde, washed with cacodylate buffer and exposed for 3 h at 31°C to the following enzymes: cellulase (Sigma) 4 mg/ml in 0.2 M sodium acetate at pH 4.5, and exo-β-1,3 glucanase from the basidiomycete QM 806 (Bauer et al., 1972), kindly supplied by Dr. Bauer, Nestlé Comp., at 4 mg/ml in 0.2 M sodium acetate of pH 5.5 Control experiments without enzymes were performed by incubating prefixed germlings in 0.2 M sodium acetate at pH 4.5.

Following incubation, the germlings were washed with buffer and prepared for electron microscopy as described above.

In vivo-Effect of Enzymes

To check the effect of enzymes on germination, 4 mg/ml of either cellulase or exo-β-1,3-glucanase were added to the germination medium before or during cyst germination. The effect of these enzymes was checked microscopically.

Turgor Pressure

To determine the osmotic pressure of the cells, germlings were sedimented for 10 min at 1000 × g and homogenized without addition of any liquids in a Braun Sonifier 300 for 2 min. The osmolarities of the homogenate and the germination medium at various dilutions were determined by measuring the freezing point depression in an osmometer (Knauer, Berlin).

RESULTS

Extracellular Enzymes

A clue to the enzymes likely to be present in apical vesicles was obtained by determining the enzymes present in the culture filtrate. The enzyme activities detected in the filtrate are shown in Table 1. UDPG-transferase, α-mannosidase and β-galactosidase activities were insignificant.

Table 1
Activities of several extracellular enzymes of *Phytophthora palmivora*

Enzyme	Activity[a]
β-1,3-Glucanase	12.4
β-Glucosidase	52.0
Acid phosphatase	2.8
Alkaline phosphatase	4.0

[a] Arbitrary units/mg protein
See "Materials and Methods" for details

Table 2. Incorporation of UDPG-^{14}C into walls
A. Radioactivity incorporated into walls of live germinating cysts (cpm/mg polysaccharide)

	Not boiled	Boiled (control)
Expt. 1	420	16
Expt. 2	6690	14
Soluble in boiling 1 n HCl	77.5%	
Soluble in boiling 1 n NaOH	5.5%	
Residue	17.5%	

B. Radioactivity incorporated into isolated cell walls (cpm/mg protein)

	Not boiled	Boiled (control)
Expt. 1	7000	250
Expt. 2	11100	5200
Expt. 3	1550	370
Soluble in boiling 1 n HCl	85.0%	
Soluble in boiling 1 n NaOH	8.0%	
Residue	7.0%	

Wall and cysts were incubated for 3 h with substrate; the results were adjusted to incorporation time of 1 h. The controls were boiled prior to incubation

Incorporation of ^{14}C-UDPG into Hyphal Walls

Germinating cysts were incubated in the presence of UDPG-(^{14}C) and the hyphal wall subsequently isolated (see Methods). Considerable radioactivity was incorporated into the wall, more than 20% of which was insoluble in boiling HCl (Table 2A). When isolated cell walls were incubated in the UDPG-transferase assay solution (see "Material and Methods") label was again incorporated into wall material (Table 2B). From this we concluded that at least part of the hyphal

Table 3. Specific activities and percentages of sedimentation of some enzymes in fractions obtained by differential centrifugation of a homogenate of germinating cysts

Enzyme	Sediment 1–8000 × g		Sediment 8–23000 × g		Sediment 23–50000 × g	
	Specific activity	% sedimentable activity	Specific activity	% sedimentable activity	Specific activity	% sedimentable activity
UDPG-transferase	1200[a]	85	365[a]	10	310[a]	5
β-1,3-Glucanase	0.16	53	0.18	22	0.36	25
β-Glucosidase	0.7	61	0.78	24	0.80	15
Acid phosphatase	0.39	75	0.24	16	0.22	9
Alkaline phosphatase	0.19	65	0.20	20	0.23	15
α-Mannosidase	0.75	75	0.53	19	0.31	6

[a] cpm/mg protein = unless otherwise indicated specific activity is expressed in arbitrary units/mg protein

UDPG-transferase activity is associated with the wall.

Intracellular Location of Enzymes

The homogenate was separated into various fractions using differential and density gradient centrifugation and the distribution of enzymes determined.

a) Differential Centrifugation. The distribution of some enzyme activities between three fractions is shown in Table 3. The majority of sedimentable UDPG-transferase activity was found in the 1–8000 × g fraction, and might be partly associated with the wall fragments found in this fraction. However, nuclei, mitochondria, lipid vacuoles, rough ER and occasional vesicles and membrane fragments were also present. The UDPG-transferase activity in the other two sediments presumably represents intracellular activity and the 8–23000 × g fraction contained vesicular membranes, microtubuli, vacuolar membranes, structures resembling dictyosomes and mitochondrial fragments.

b) Density Gradient Centrifugation. The 8–23000 × g fraction separated into three bands following centrifugation on a Urografin gradient (Fig. 1). The majority of UDPG-transferase activity was associated with band 1 (Fig. 1). The distribution of β-glucanase was quite different, the majority of activity occurring in bands 2 and 3. The mitochondrial enzymes, malate dehydrogenase and citrate synthetase, were found in bands 1 and 3 (Fig. 1). Intact mitochondria were present in band 1 and the activities in band 3 presumably reflect the presence of mitochondrial fragments and possibly some enzyme release. The acid hydrolases (β-glucosidase, α-mannosidase and acid phosphatase) were active in all three bands. Alkaline phosphatase and $NADH_2$-ferricyanide reductase activities were not significant in band 2, and the alkaline phosphatase was most active in band 1. Low activities of AMP-ase and TPP-ase, thought to be markers for plasma membrane and dictyosomes (Cheetham et al., 1970), were detected in all three bands. The upper portion of the band containing the high UDPG-transferase activity from Urografin gradients (band 1) was centrifuged through Ficoll step-gradients (see "Material and Methods"). Three bands were again obtained and the most dense (band A, ϱ 1.09) contained the majority of the UDPG-transferase activity that had initially been loaded (Fig. 2). The fraction contained many vesicles and membrane fragments, including rough and smooth ER. The membrane fragments did not appear to be plasma membrane. Some contamination with vacuolar fragments was apparent. The specific activity of UDPG-transferase in band A was consistently high and comparable to activity found in wall preparations (Table 4). The acid hydrolases, α-mannosidase and β-glucosidase, were also found in band A. However, their activity was only a fifth of that found in Urografin gradients. Alkaline phosphatase activity was also associated with band A. The relatively low β-glucanase activity loaded onto Ficoll gradients again peaked in the upper, less-dense band.

Histochemistry

a) Alkaline Phosphatase. The lead phosphate precipitate was observed mainly in mitochondria, rough and smooth ER and in the proximal areas of the dictyosomes (Fig. 3). In particular reaction product was present in the small vesicles that derive from the ER and fuse to form a cisterna at the proximal pole of the dictyosome. Controls treated in the absence of substrate gave similar results. However, product was restricted to mitochondria if the tissue was heated prior to incubation. Apparently adequate substrate was available in vivo to permit the reaction.

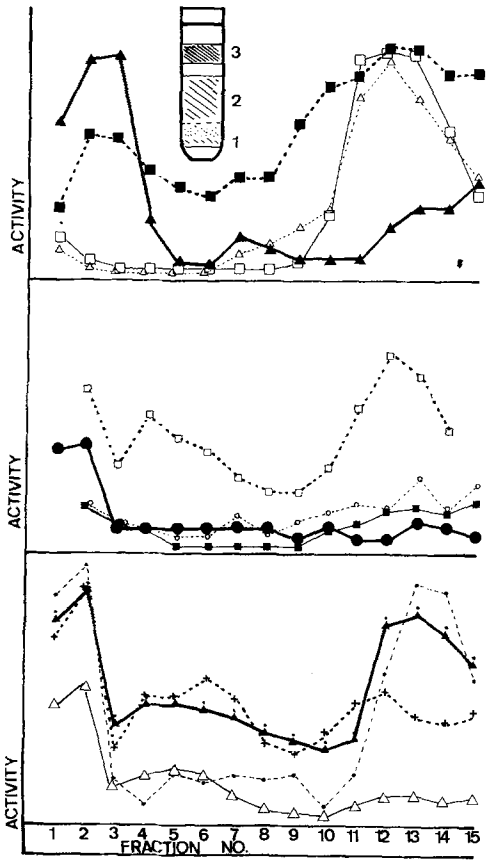

Fig. 1. Distribution of enzyme activities among fractions from the Urografin gradient (see "Material and Methods"). Fraction 15 = top. ▲——▲, UDPG-transferase; ■----■, exo-β-1,3-glucanase; □——□, malate-dehydrogenase; △----△, citrate synthetase; □----□, $NADH_2$-reductase; ○----○, AMPase; ■——■, TPPase; ●——●, alkaline phosphatase; ▲——▲, acid phosphatase; •-----•, protein; +----+, β-glucosidase; △——△, α-mannosidase

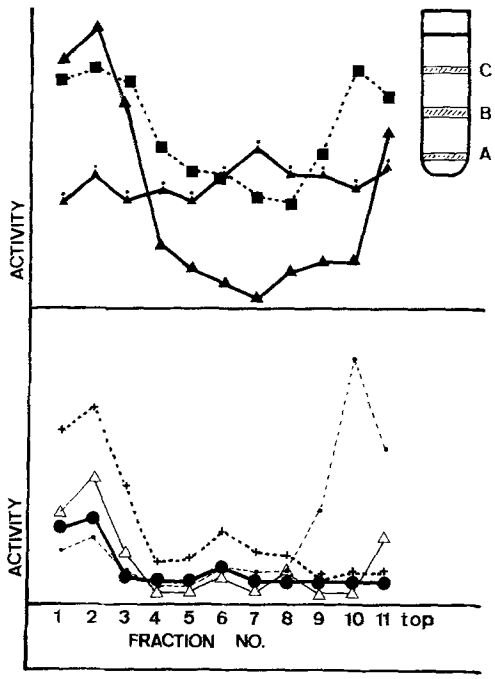

Fig. 2. Distribution of enzyme activities among fractions of Ficoll gradient (see "Material and Methods"). ▲——▲, UDPG-transferase; ■----■, exo-β-1,3-glucanase; ▲——▲, acid phosphatase; ●——●, alkaline phosphatase; •-----•, protein; +----+, β-glucosidase; △——△, α-mannosidase

Table 4. UDPG-transferase activities obtained from different fractions of germinating cysts

Fraction	cpm/mg protein
Isolated cell walls	1350; 4450; 5100
Sediment 1–8000 × g	1250
Fraction A of Ficoll gradient	3700; 6000; 11250

b) Acid Phosphatase. Reaction product was found in dictysomes, usually restricted to the middle cisternae. The inner side of the "crystalline" vacuole membranes were also strongly stained (Fig. 4). Boiled controls showed no activity.

c) Thiéry's Polysaccharide Reaction. In germinating cysts silver particles were observed in the plasma membrane, in dictyosomes and membranes of apical vesicles (Figs. 5 and 6), as well as in lipid and 'cristalline' vacuoles. The contents of the vesicles also reacted, although more weakly than the vesicle membranes. Hyphal walls also reacted weakly. Except for normal background, no silver grains were found in control preparations lacking the coupling reagent.

Enzymatic Wall Degradation

Cell walls of prefixed germlings treated with cellulase do not show any distinct difference from controls prepared in the absence of enzyme. The exo-β-1,3-glucanase treatment, however, leads to an almost total removal of the outer layer while the inner layer remains as a thin, compact structure. With the same treatment the wall at the hyphal apex is almost completely removed (Figs. 7 and 8). This indicates that the wall at the apex is different from the lateral walls and its integrity lost by the action of an exo-β-1,3-glucanase.

In vivo-Effect of Enzymes

When cellulase or β-glucanase is added to germinating cysts, the germ tubes at first stop growing. Germ tube extension is renewed after about 30 min. In the presence of cellulase the cells grow normally. In the presence of β-glucanase, however, numerous protru-

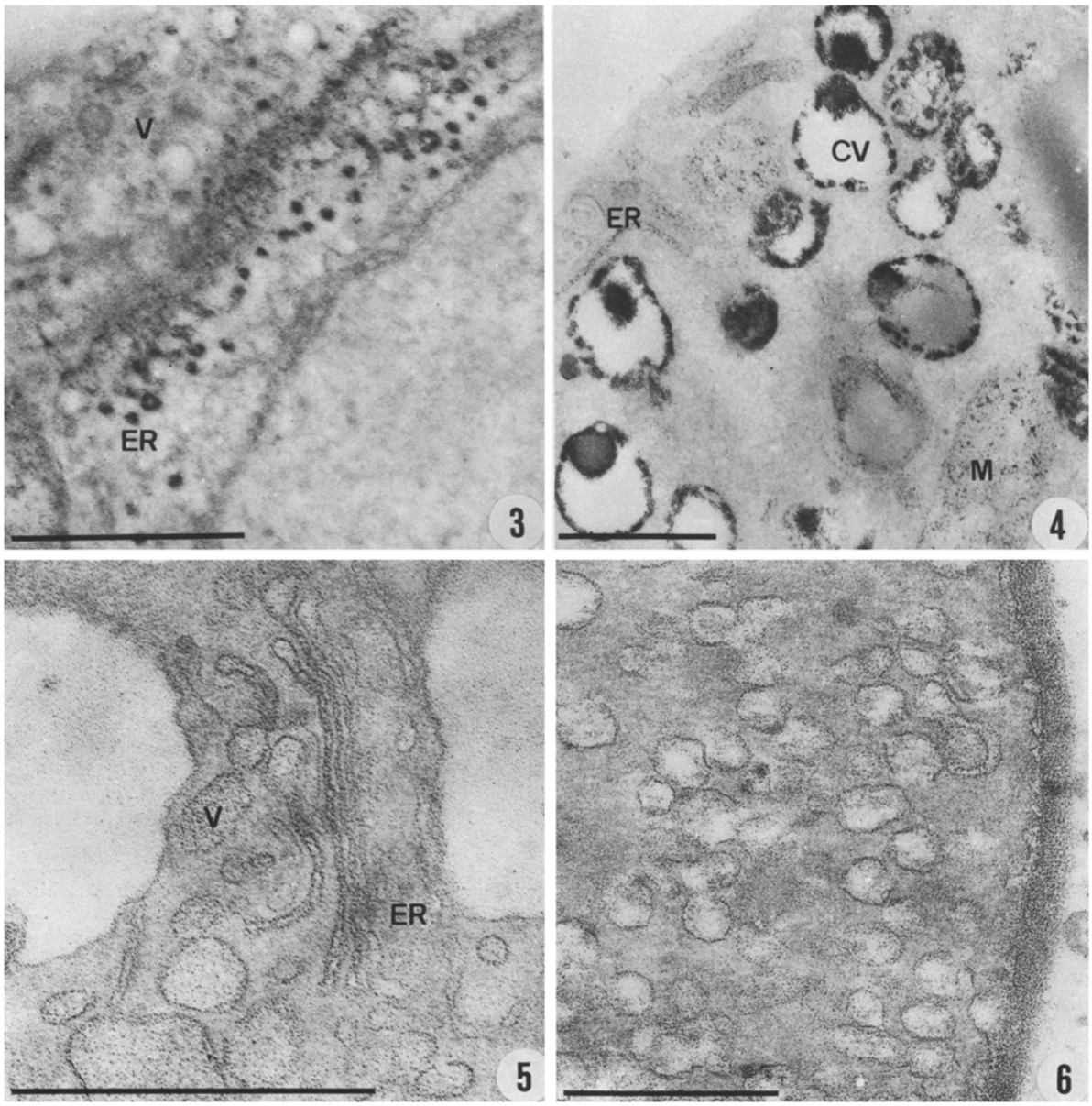

Fig. 3. Alkaline phosphatase reaction in the region of a dictyosome of a germinating cyst. The proximal side of the dictyosome which is adjacent to the endoplasmic reticulum (*ER*) stains heavily, while the dictyosome vesicles (*V*) at the distal side are largely unstained. × 34000

Fig. 4. Germinating cyst subjected to acid phosphatase reaction. A particularly strong reaction is exhibited by the 'cristalline' vacuoles (*CV*), less so by the endoplasmic reticulum (*ER*) and the mitochondria (*M*). × 22000

Fig. 5. Thiéry's polysaccharide reaction of a germinating cyst. Reaction product increases from the proximal (*ER*) to the distal face where the vesicles (*V*) are located. × 54000

Fig. 6. Thiéry's polysaccharide reaction of a cyst at very early germination. The cyst wall and the numerous vesicles that are massed below it are markedly stained. × 31000

sions and irregular hyphal branches may form (Fig. 9). Further, branches may develop from the apex and very close to the cyst wall which is not normally the case in controls (Fig. 10). Multiple germ tubes may also develop from a single cyst.

Turgor Pressure

The result of these measurements is shown in Figure 11. It gives the osmotic pressure from a series of dilutions obtained from the homogenized germlings and the

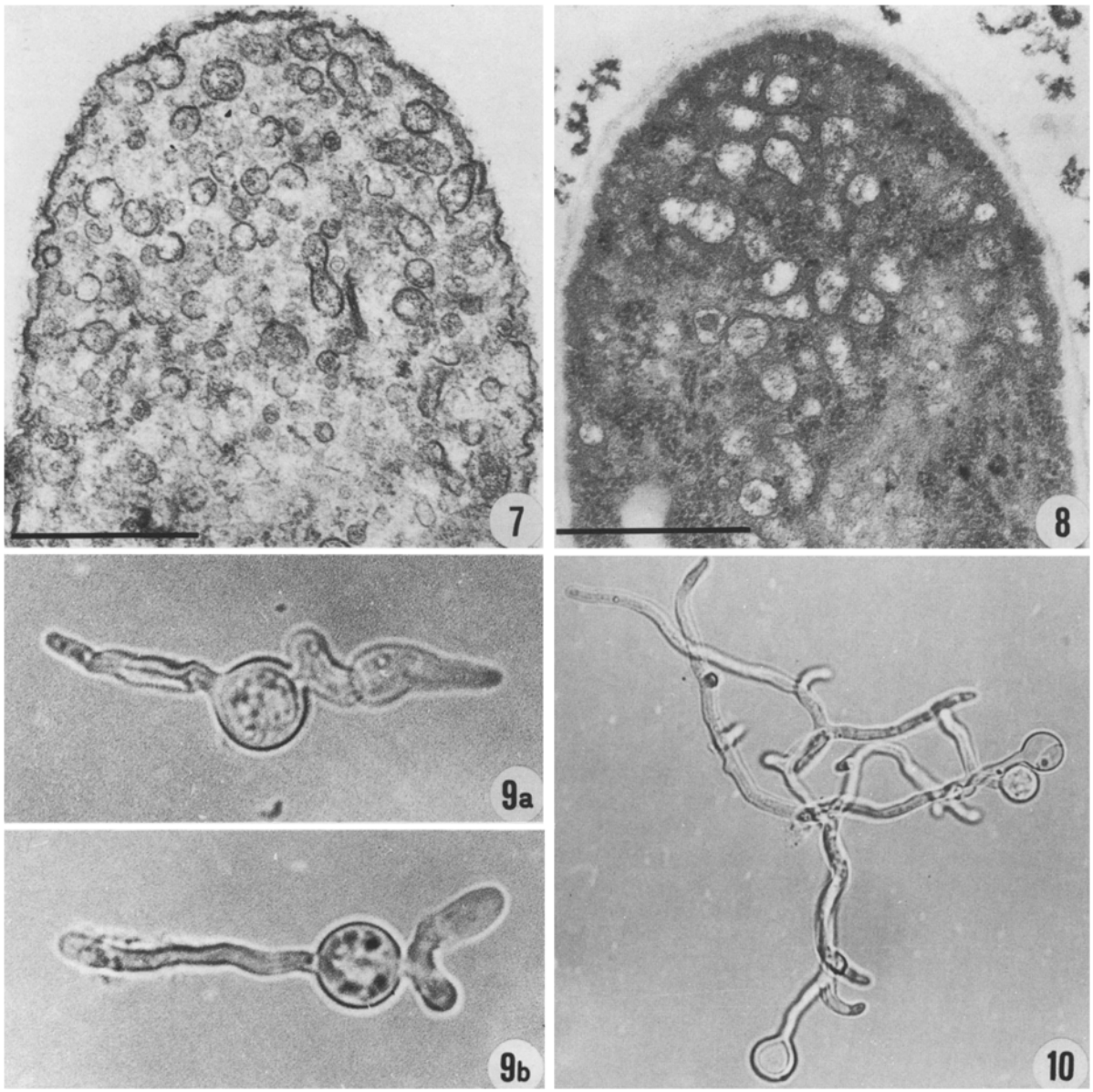

Fig. 7. Longitudinal section through hyphal tip following treatment with β-1,3-glucanase. The hyphal wall is largely missing. $\times$ 28 000

Fig. 8. Control experiment without glucanase treatment: note the presence of a rather smooth, continuous wall covering the entire hyphal apex. $\times$ 28 000

Fig. 9a and b. Cysts that have germinated in the presence of β-1,3-glucanase: note the swollen germ tubes with unusual branching close to the cyst which is hardly observed under normal conditions. $\times$ 340

Fig. 10. Control experiment with cysts germinating in the absence of glucanase. Compare with Figure 9. $\times$ 240

incubation medium. The difference in osmolarities between the medium (0.25) and the cell homogenate (0.38) corresponds to a pressure of 1.8 at which represents the turgor of the germinating cysts.

DISCUSSION

The turgor of germinating cysts of *Phytophthora palmivora*, i.e. that amount of the osmotic pressure of the protoplast which exceeds the osmotic pressure of the surrounding incubation medium, amounts to approximately 1.8 at. This value is considerable but lower than that obtained from some other fungal systems: the corresponding values for *Neurospora crassa* are 12.4 at (Robertson and Rizvi, 1968), for *Mucor hiemalis* 4-11 at and for *Aspergillus wentii* 12-18 at (Adebayo et al., 1971). It should be considered, however, that the various results were obtained with

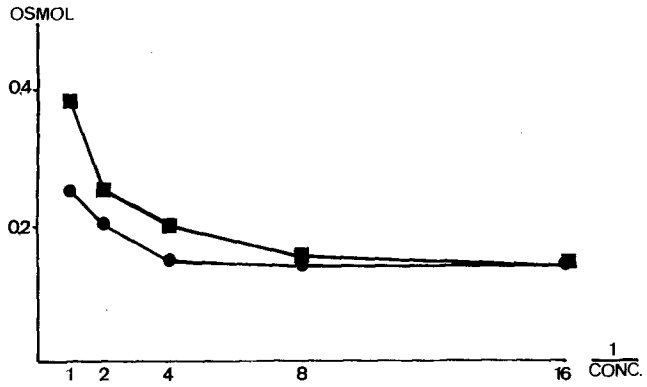

Fig. 11. Osmolarity of homogenate and germinating medium at different dilution rates (see "Material and Methods"). The difference of 0.13 osmolarities at zero dilution corresponds to 1.8 at. ■——■, homogenate; ●——●, germination medium

different techniques and are thus difficult to compare. The main fact emerges that in *P. palmivora*, too, the turgor of the germ tube is likely to provide the major thrust for extending the hyphal apex, as has been proposed for fungal systems generally (Robertson, 1968).

Germ tubes grow normally in the presence of the cellulase used but are swollen and display a strong tendency for apical branching when exposed to the β-1,3-glucanase. In addition, thin sections show that most, if not all of the stainable wall material at the hyphal apex is removed by this treatment. Further behind the apex the lateral walls seem to be mostly lacking the amorphous outer layer while retaining the inner, fibrillar parts, corroborating the results of Hunsley (1973) with laminarase (a β-1,3-glucanase) and hyphae of *P. parasitica*. Since we have shown that *P. palmivora* produces considerable amounts of extracellular β-1,3-glucanase (Holten and Bartnicki-Garcia, 1972, found hardly any), the combined results favor the idea that weakening of the apical wall by the removal of β-1,3-glucan leads to an uncontrolled mode of hyphal growth as expressed by irregular hyphal swellings and apical branching. Hence, exo-β-1,3-glucanases are implicated in the control of normal hyphal tip growth in *Phytophthora*. In *Schizosaccharomyces* endoglucanases but not exoglucanases cause considerable cell wall lysis (Fleet and Phaff, 1974). In *P. palmivora*, the presence of endoglucanases has not been investigated.

We have confirmed the results of Wang and Bartnicki-Garcia (1966) that the major portion of the sedimentable UDPG-transferase activity is localized in the wall fraction. However, our results show a higher fraction (≥15%) of the activity bound to membranes than theirs (9%). A high percentage of wall bound synthetases has also been observed with chitin forming fungi such as *Mucor rouxii* (McMurrough et al., 1971) or *Neurospora* crassa (Mishra and Tatum, 1972).

The most striking result obtained from the density gradient studies was the consistent separation of β-glucanase and UDPG-transferase enriched fractions on the gradients. There are at least two explanations for this observation. First, it could be argued that both β-glucanase and UDPG-transferase are transported in dictyosome derived vesicles whereby one or the other enzyme could be temporarily inactivated during the vesicle transport. This idea would adhere to the notion that all vesicles fusing with the plasma membrane are dictyosome derived and of basically one type only, any difference in distribution of enzyme activities in the biochemical gradients being the result of differential enzyme inactivation.

The second alternative is more appealing. It states that there are at least two types of vesicles, one containing entirely or predominantly the glucanase, the other the transferase. One type of vesicle would most likely be identical with the dictyosome-derived, larger vesicle found in great numbers at the growing hyphal tip (Girbardt, 1969; Hemmes and Hohl, 1969; Grove and Bracker, 1970) and staining faintly for polysaccharide. The second type could conceivably be identical with the less numerous small vesicles observed in the same area (Hemmes and Hohl, 1969). The origin of this latter type of vesicle is not clear. It might be derived from dictyosomes or directly from the endoplasmic reticulum. Since we found the UDPG-transferase enriched fractions were relatively rich in membranes resembling rough and smooth endoplasmic reticulum, this enzyme could well be ER associated and transported in ER-derived vesicles. While it is not clear how such vesicles could fuse directly with the plasma membrane, ER—as well as Golgi—derived vesicles are apparently capable of transporting membrane components and secretory products in plants (Bowles and Northcote, 1972; Morré et al., 1971; Morré and Mollenhauer, 1974; Roland, 1973) and animal cells (Ross and Benditt, 1965). Further, cellulose synthetase is transported to the pea wall: protoplast interface in both Golgi and ER vesicles (Shore and Maclachlan, 1975; Shore et al., 1975).

Plasma membrane and dictyosome vesicle membranes react strongly with the Thiéry reagent for polysaccharides. The dictyosomes themselves display a gradient of diminishing reactivity towards their proximal face while the contents of the apical vesicles react only faintly. Either the vesicles do not contain much polysaccharide or their degree of polymerization is too low for a marked reaction (Geyer, 1973). Apical vesicles in several other hyphal systems have

been shown to react strongly with the polysaccharide stain (Heath et al., 1971; Dargent et al., 1974; Dargent, 1975).

In addition to β-glucanase the two acid hydrolases, β-glucosidase and acid phosphatase, were found extracellularly. However, the distribution of the latter two enzymes in gradients differed somewhat from β-glucanase. This may be because they are associated with vacuoles as well as vesicles transporting them to the wall. The "crystalline" or "finger-print" vacuoles (Hemmes and Hohl, 1971), for example, stain strongly for acid phosphatase (Meyer, 1975). Whether in fact β-glucosidase and acid phosphatase are transported to the wall in the same vesicles as β-glucanase cannot be ascertained.

Alkaline phosphatase activity is present in all bands, but is rather more enriched in the denser fractions containing UDPG-transferase activity. Since the enzyme is found extracellularly it may be transported in the same vesicles as the UDPG-transferase. However, the histochemical results are difficult to reconcile with this proposal, unless the enzyme is inactive during transport to the wall. If so, this would account for the observation that histochemical reactivity is restricted to the proximal region of the dictyosome and the ER membranes and transition vesicles associated with it. Interestingly, in the only other hyphal tip region checked for the presence of alkaline phosphatase, that of *Achlya bisexualis* (Dargent, 1975), the enzyme activity is concentrated on the distal side of the dictysomes and in the dictyosome derived apical vesicles.

In conclusion, we postulate the presence of two different intracellular routes by which enzymes are transported to the wall. Two types of vesicles may be involved, one containing β-glucanase and the other UDPG-transferase and possibly alkaline phosphatase. The contamination of all fractions by vacuole fragments makes it at present difficult to predict whether extracellular hydrolases are transported in one or the other type, or by a separate mechanism altogether.

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