Effect of phytoalexins on hyphal growth and β-glucanases of Phytophthora infestans

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Abstract

Phytophthora infestans excretes an endo-β-1,3-, an endo-β-1,4-, and a β-1,3-glucanase (laminarinase), a β-1,6-glucosidase and possibly small amounts of a β-1,4-glucosidase. Ether extracts from the infected resistant cultivar Eba but not from the susceptible Bintje inhibited growth of the parasite. Solavetivone and rishitin, two phytoalexins, and the steroid glycoalkaloid tomatine inhibited growth of the fungus and also activities of some of the fungal glucanases, whereas phytuberin, another phytoalexin, and the two phenolic compounds scopoletin and chlorogenic acid inhibited neither fungal growth nor fungal glucanases. The phytoalexin lubimin strongly reduced fungal growth but did not reduce the activities of any of the fungal glucanases tested. A potential role for host derived fungal glucanase inhibitors as factors of resistance in the *Phytophthora*-potato system is discussed.

Introduction

According to Bartnicki-Garcia (4), hyphal tip growth in fungi is maintained by a balance of wall-synthesizing and wall degrading processes. The wall of *Phytophthora* consists mainly of β-glucans (3, 5, 22). Fungal β-glucanases capable of degrading such glucans might thus be essential for hyphal tip growth, and their inhibition could conceivably disturb the balance between synthesis and degradation of wall material.

In potato tubers of the resistant cultivar Eba, *P. infestans* is restricted to the site of penetration, and haustoria are surrounded by electron-translucent encapsulations usually not present in the susceptible cultivar Bintje (13). The chemical composition of these wall appositions may be very complex (see 1), but in potato tissue two of the main components are callose or a related β-1,3-glucan and cellulose (9, 11, Hohl & Hächler, unpublished).

It has been argued (13) that in the compatible host-parasite relationship β-glucanases produced by the pathogen might prevent the formation of these glucan deposits. On the other hand, a potential host-derived glucanase inhibitor might not only permit encasements to form but also reduce intercellular growth of the parasite, two phenomena observed to occur in the incompatible plant pathogen combination. We have, therefore, started to investigate the types of glucanases produced by phytophthoras and the presence of glucanase inhibitors in the host tissue.

This paper deals with the identification of various extracellular β-glucanases of *P. infestans* and their activity in the presence of phytoalexins and other substances known to be produced by hosts belonging to the *Solanaceae*. In addition, the effect of these same compounds on fungal growth is also described.

Materials and methods

For growth assays, *Phytophthora infestans* Mont. (de Bary) strain S (race 4) was grown at 24 °C on the synthetic P-1L agar medium (12) in

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90 mm plastic Petri dishes. To obtain culture filtrates the fungus was grown for 14 d at 16 °C in 250 ml Erlenmeyer flasks, containing 50 ml of P-1 (12) medium. Culture filtrate was centrifuged for 30 min at 20,000 g at 4 °C, the supernatant concentrated by ultrafiltration (Amicon Corp., UM 10 membrane), dialyzed in 10 mM phosphate buffer of pH 6.5 overnight, and then used for enzyme assays. The protein content was determined according to Lowry *et al.* (17).

For production of sporangia *P. infestans* was grown for 18 d at 16 °C on 1.5% agar containing 30 g homogenized kidney beans per liter. The sporangia were collected in sterile water and allowed to release zoospores at 4 °C. Discs of peeled and surface sterilized potato tubers of the cultivars Bintje (r, susceptible) and Eba (R3, resistant) were put into sterile moist Petri dishes, inoculated with the zoospore suspension (ca. 10^5/ml) and incubated in a moist chamber at 18 °C. Controls were treated likewise but covered with sterile water only. After 4 d, the top 2 to 3 mm of the tuber discs were cut off, weighed and homogenized in a small amount of distilled water. Following the method of Horikawa *et al.* (15), the resulting suspensions were extracted three times with an equal volume of diethyl ether, the combined extracts concentrated under reduced pressure at 30 °C and washed with a small volume of distilled water. After drying the extracts in a stream of nitrogen, each residue was dissolved in distilled water containing a small amount of ethanol. For identification the extracted compounds were chromatographed on silica gel thin layer plates (Merck) with methanol-chloroform (5:95) as solvent (21). The sesquiterpenes were detected by spraying with 5% phosphomolybdic acid in ethanol and identified by comparing them with the co-chromatographed standards lubimin, phytuberin, rishitin, and solavetivone.

For enzyme inhibition studies, chlorogenic acid, scopoletin (Fluka) and tomatine (Sigma) were used in the experiments in addition to lubimin, phytuberin, rishitin, and solavetivone. Tomatine was used instead of the potato alkaloids solanine and chaconine which were not available.

Enzyme assays

All substrates were dissolved in citrate phosphate buffer (McIlvaine) containing 3 mM sodium azide. After addition of phytoalexins or tissue extracts dissolved in 100 µl 50% ethanol (similar amount of ethanol also added to control) and enzyme (control: heat-denatured proteins), the solutions were incubated on a shaker at 38 °C. The various assays were carried out as follows:

β-1,4-, and β-1,6-glucosidase

0.7 ml of 0.05 M cellobiose or gentiobiose (Fluka), respectively, and 0.3 ml of enzyme solution were incubated for 24 h. The reaction was stopped by immersion of the vials in boiling water, and the glucose produced was measured spectrophotometrically, with hexokinase and glucose-6-phosphate dehydrogenase (glucose-test, Boehringer). Hexokinase and glucose-6-phosphate dehydrogenase used in this procedure were not affected by the phytoalexins.

β-1,3-glucanase

Laminarin (Sigma) was purified according to the method described by Barras and Stone (2) and 10 mg was dissolved in 1.5 ml of buffer and incubated for 24 h with 0.5 ml of the enzyme solution. The reaction was terminated by immersion of the tubes in boiling water, and the amount of carbohydrates was determined with the anthrone reagent: 5 ml 0.1% anthrone in 72% sulfuric acid were allowed to react with a test sample for 10 min by immersion of the tubes into boiling water. The reaction was stopped in an ice bath and the extinction measured at 620 nm (D. Rast, personal communication).

Endo-glucanases

The method of Clarke and Stone (8) was used to produce sodiumcarboxymethylpachyman from pachyman, a linear β-1,3-glucan of *Poria cocus* Wolf. The enzyme assay was done according to the method of the same authors.

0.5% sodium 4-carboxymethylcellulose (Fluka) and 2% pustulan (Calbiochem), a linear β-1,6-glucan, were the substrates for the endo-β-1,4- and the endo-β-1,6-glucanase, respectively. Viscosity of the solutions was measured in a 'Cannon-Ubbelohde Viscometer', and the enzyme activity expressed as reduction of efflux time (in %) compared to the non-hydrolysed substrate of the control.

Table 1. Mycelial growth of *P. infestans* in 3 different media containing either the glucosides cellobiose, gentiobiose, or the glucans laminarin, cellulose or pustulan as carbon source. Control (1) did not contain any glucan or sugar and control (2) contained either 10 g of glucose/l (medium I and II) or 2 g/l (medium III). The cultures were grown at 24 °C for 21 d in Erlenmeyer flasks containing 10 ml of medium I or II and for 30 d in 50 ml of medium III, respectively. Growth is expressed as mg dry weight per 10 ml of medium. nt = not tested.

Medium	Components (in g/l dist. water)				Mycelial dry weight						
					Control 1	Control 2	Cellobiose (β-1,4)	Gentiobiose (β-1,6)	Laminarin (β-1,3)	Cellulose (β-1,4)	Pusti (β-1,
I	KH_2PO_4	0.5	$CaCl_2$	0.02	0.5	6.1	0	9.0	0.5	3.0	2.7
	K_2HPO_4	0.5	Aspargine H_2O	0.1							
	$NaNO_3$	0.2	Thiamine HCl	0.001							
	$MgSO_4$ $7H_2O$	0.5	carbon source	10.0							
II	NH_4NO_3	2.0	$CaCl_2$	0.02	0	3.6	0	5.2	0	0	0
	KH_2PO_4	0.43	Thiamine HCl	0.001							
	Na_2HPO_4 $2H_2O$	0.56	carbon source	10.0							
	$MgSO_4$ $7H_2O$	0.5									
III	Hohl's P-1 Sucrose is replaced by either cellobiose 2.0 or laminarin 10.0				5.6	12.3	12.0	nt	16.7	nt	nt

Results

Mycelial growth on various carbon sources

The following carbohydrates were tested for their ability to support growth of *P. infestans:* laminarin, cellulose, pustulan, cellobiose, gentiobiose. They all consist of β-linked glucose units only. Tested in three different basal media, the resulting fungal growth differed very much (Table 1). With sodium nitrate and asparagine as nitrogen sources and either cellobiose or laminarin as carbon source, no or very little fungal growth took place. In the same medium but with gentiobiose as carbon source abundant mycelial growth occurred. Growth was about one third of that with gentiobiose, when either cellulose or pustulan was used as the carbon source. On the other hand, no growth was observed in a minimal medium containing ammonium nitrate as the only nitrogen source and either laminarin, cellulose or pustulan as a carbon source. In the rich medium P-1 (12) good fungal growth was registered after replacing saccharose by cellobiose or laminarin. These results indicate 1) that *P. infestans* can excrete enzymes necessary to split β-1,3, β-1,4 and β-1,6 glucans into smaller units and 2) that these smaller molecules and disaccharides were further split and/or absorbed into the mycelium. Finally (3), synthesis of the enzymes varies with the nutritional conditions.

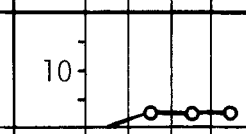
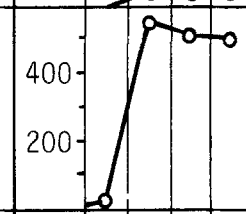
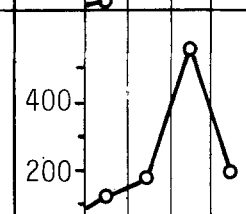
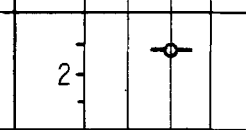
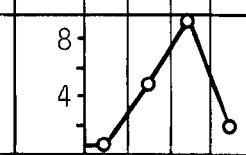
Occurrence and activity of extracellular β-glucosidases and β-glucanases of P. infestans

To determine the approximate pH-optima, the enzyme activities were measured from pH 4 to 7. As summarized in Table 2, activity of the β-1,6-glucosidase was highest at pH 5 whereas that of β-1,3-glucanase (laminarinase) and endo-β-1,4-glucanase was optimal at pH 6. The β-1,4-glucosidase was not very active at any of the pH-values tested, and no endo-β-1,6-glucanase activity was detected. Because of shortage of pachyman, the pH-optimum of the endo-β-1,3-glucanase could not be determined and the enzyme test was always carried out at pH 5.5. These results demonstrate the presence of extracellular fungal enzymes required to digest β-1,3-, β-1,4- and also β-1,6-glucans.

Hyphal growth in the presence of extracts from potato tubers

In these experiments ether extracts from 0.5 g fr. wt. of tubers/ml of medium were used. Extracts from non-infected tubers increased rather than

Table 2. Extracellular β-glucosidases and β-glucanases of *P. infestans:* relationship of specific activity to pH.

Enzyme	Substrate	Specific Enzyme Activity
β-1,4 glucosidase	cellobiose	 μM glucose / mg protein · h
β-1,6-glucosidase	gentobiose	 μM glucose / mg protein · h
β-1,3-glucanase	laminarin	 μM carbohydrates* / mg protein · h *glucose equivalent
endo-β-1,3-glucanase	carboxymethyl-pachyman	 red. efflux time (%) / mg protein · h
endo-β-1,4-glucanase	carboxymethyl-cellulose	 red. efflux time (%) / mg protein · h
endo-β-1,6-glucanase	pustulan	no detectable activity

reduced hyphal growth of *P. infestans* (Fig. 1). In the presence of ether-soluble compounds from infected susceptible tubers no significant inhibition of growth was detectable, whereas extracts from resistant infected tubers reduced hyphal growth by 52% (Fig. 1).

As shown with thin layer chromatography, diethyl ether extracts from non-infected potato tubers of Eba contained only traces of solavetivone whereas no solavetivone was detected in extracts of tubers of the susceptible cultivar Bintje. On the other hand, infected tubers of both cultivars contained lubimin, phytuberin, rishitin and solavetivone; all occurred in considerably smaller amounts in the susceptible cultivar than in the resistant one. Furthermore, the extracts from both cultivars also contained two (before infection) or three (after infection) unidentified, only slightly lipophilic (Rf 0.025–0.1) but phosphomolybdic acid-reactive compounds.

Effect of phenolic and terpenoid compounds on the mycelial growth of P. infestans

Several compounds occurring in healthy and/or *P. infestans* infected potatoes or tomatoes were tested for their effect on hyphal growth of the pathogen. The experimental results are shown in

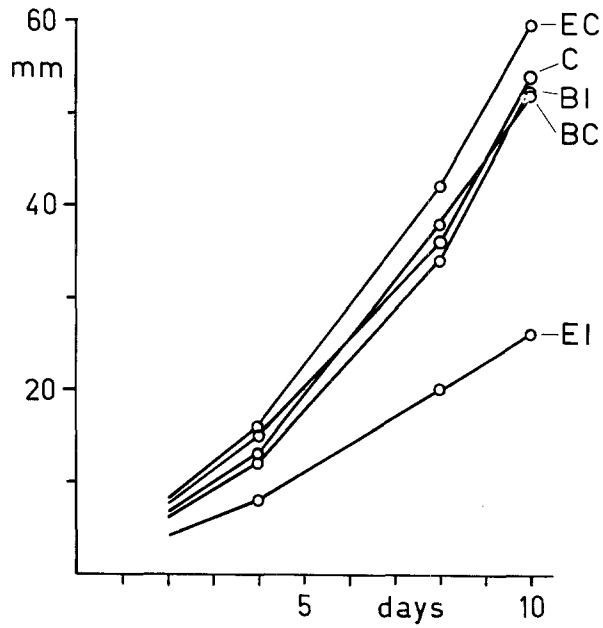


Fig. 1. Hyphal growth of *P. infestans* on P-1L agar at 24 °C containing ether extracts (from 0.5 g fr. wt of tuber tissue/ml medium) from potato tubers: control (C) with no ether extract added; susceptible cultivar Bintje (BC), non-infected; susceptible cultivar Bintje (BI), infected; resistant cultivar Eba (EC), non-infected; resistant cultivar Eba (EI), infected. Growth is expressed as mm of colony diameter.

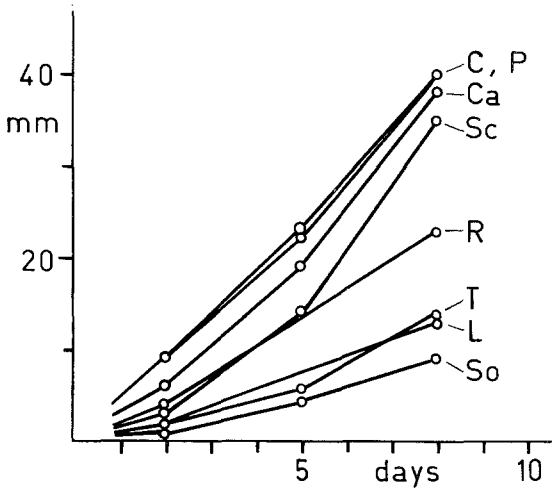


Fig. 2. Hyphal growth of *P. infestans* at 24 °C on P-1L agar containing one of the following compounds: Chlorogenic acid (Ca), scopoletin (Sc) or tomatine (T), each at 10^{-4} M and lubimin (L), phytuberin (P), rishitin (R) or solavetivone (So), each 50 µg/ml medium; control (C) without additional compounds. Growth is expressed as mm of colony diameter.

Figure 2. At concentrations of 50 µg/ml, there was a marked inhibition by all terpenoids except phytuberin. After 8 d solavetivone had reduced hyphal growth by 80%, lubimin and the steroidglycoalkaloid tomatine by 68% and rishitin by 40%. At the same time, microscopical observations showed an increase in hyphal branching as a result of the treatment with terpenoids or tomatine. The two phenolic compounds, tested at 10^{-4} M concentration, had little inhibitory activity resulting in less than 15% reduction of hyphal growth.

Influence of phytoalexins on extracellular fungal β-glucosidases and β-glucanases

The influence of the phenolic and terpenoid compounds used in the previous experiment was tested on the activity of the enzymes produced by *P. infestans*. The substances were used at 50 µg or 250 µg/ml, respectively. The results are shown in Figures 3–6:

(1) Tomatine (10^{-4} M) and solavetivone (250 µg/ml), two very effective growth inhibitors, both significantly reduced the activity of the endo-β-1,4-glucanase (Fig. 6). Solavetivone also reduced the endo-β-1,3-glucanase activity (Fig. 5). A lower concentration (50 µg/ml) of solavetivone did not show significant effects.

(2) 250 µg/ml of rishitin greatly reduced the endo-β-1,4-glucanase (Fig. 6). Lower concentrations of rishitin have not been tested due to shortage of material.

(3) The phenolic compounds and phytuberin, which had little effect on growth, also did not inhibit the activity of the enzymes tested.

(4) None of the compounds inhibited β-1,6-glucosidase (Fig. 3).

(5) Lubimin, which strongly inhibited growth, did not inhibit any of the endo-β-glucanases.

Discussion

The strain of *P. infestans* used in this study excretes endo-β-1,3-, and endo-β-1,4-, and a β-1,3-glucanase (laminarinase), a β-1,6-glucosidase and possibly small amounts of a β-1,4-glucosidase. These enzymes are all produced in the same medium (P-1) containing sucrose as the sole carbohydrate. Some of these enzymes have been reported from other *Phytophthora* species (7, 14, 16, 18, 19).

Figs. 3–6. Influence of the following compounds on the specific activity of several extracellular β-glucosidases and β-glucanases of *P. infestans*. Control (C); scopoletin (Sc), chlorogenic acid (Ca), tomatine (T), phytuberin (P), 10^{-4} M each; solavetivone (So), lubimin (L), rishitin (R), 250 μg/ml each, except for solavetivone in Fig. 3 & 4 where only 50 μg/ml were used.

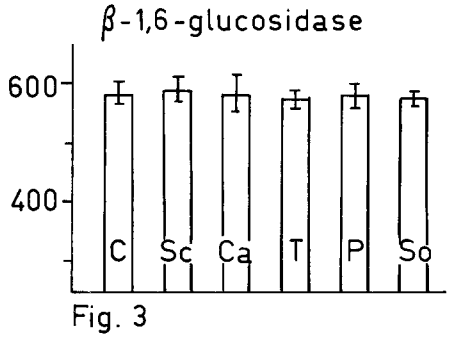


Fig. 3

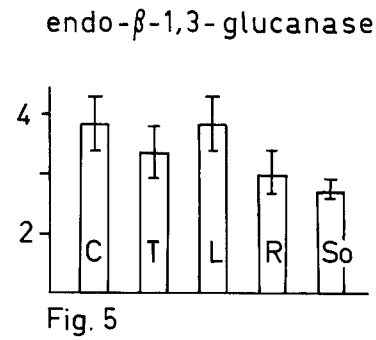


Fig. 5

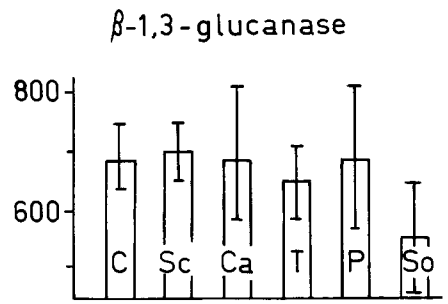


Fig. 4

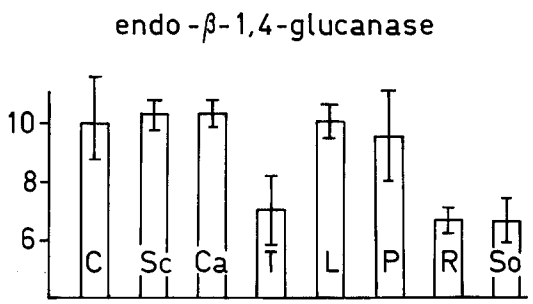


Fig. 6

Fig. 3. β-1,6-glucosidase (pH 5). Activity expressed as μM glucose/mg protein · h.

Fig. 4. β-1,3-glucanase (laminarinase) at pH 6. Activity expressed as μM reacting carbohydrates (glucose equivalent)/mg protein · h.

Fig. 5. endo-β-1,3-glucanase (pH 5.5). Activity expressed as % reduction of efflux time/mg protein · h.

Fig. 6. endo-β-1,4-glucanase (pH 6). Activity expressed as % reduction of efflux time/mg protein · h.

As a result of infection by *P. infestans* the resistant potato tuber produces fungitoxic principles such as terpenoids, steroidalkaloids and phenolic compounds which are either absent or present in ineffective quantities in uninfected resistant or infected and noninfected susceptible tubers. Even though phytuberin is known to inhibit strains of *P. infestans* (6) and coumarin and some of its derivatives at 10^{-4} M each are reported to inhibit growth of *P. capsici* (10), neither chlorogenic acid or scopoletin, both present in increased quantities in infected solanacean hosts (20), nor phytuberin showed a significant effect on hyphal growth of our strain of *P. infestans*.

Among the phytoalexins tested rishitin and solavetivone significantly inhibited the activity of the endo-β-1,4- or the endo-β-1,3- and endo-β-1,4-glucanase, respectively. This is interesting since both phytoalexins 1) reduce fungal growth, 2) are formed primarily by the resistant cultivar Eba, and 3) β-1,3. and β.1,4-glucans constitute by far the largest portion of the fungal cell wall material (3). Our results show, however, that the concentrations of phytoalexins causing even partial inhibition of these glucanases is higher than the concentrations needed to reduce hyphal growth rate by 50% or more. It should be considered, on the other hand, that even small reductions of enzyme activities might lead to great disturbances of hyphal tip growth if, as has been postulated (4) hyphal tip growth indeed is dependent on a delicate balance of wall synthesizing and wall lysing processes. Our observation that, with the exception of lubimin effective growth inhibitors also showed some effects on the activity of endo-glucanases whereas non-inhibitors did not, tends to corroborate this point.

Our results show that there are compounds produced by resistant potato cultivar that are capable of partially inhibiting some glucanases considered essential for the parasite. Clearly much more work with a variety of other host derived potential inhibitors will be needed to prove or disprove our notion (13) that host derived glucanase inhibitors might be factors of resistance in the *Phytophthora*-potato system.

Acknowledgement

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References

1. Aist, J. R., 1976. Papillae and related wound plugs of plant cells. Annual Review of Phytopathology 14, 145–163.
2. Barras, D. R. & Stone, B. A., 1969. β-1,3-glucan hydrolysis from Euglena gracilis. I. The nature of hydrolases. Biochimica Biophysica Acta 191, 329–341.
3. Bartnicki-Garcia, S., 1966. Chemistry of hyphal walls of Phytophthora. Journal of general Microbiology 42, 57–69.
4. Bartnicki-Garcia, S., 1973. Fundamental aspects of hyphal morphogenesis. In Microbial Differentiation. Ed. by J. M. Ashworth & J. E. Smith, pp. 245–267. University Press, London.
5. Bartnicki-Garcia, S. & Lippman, E., 1967. Enzymic digestion and glucan structure of hyphal walls of Phytophthora cinnamomi. Biochimica Biophysica Acta 136, 533–543.
6. Beczner, J. & Ersek, T., 1976. Fungitoxicity of phytoalexins derived from potato against mycelial growth of Phytophthora infestans. Acta Phytopathologica Academiae Scientiarum Hungarica 11, 59–64.
7. Borrod, G., 1974. Contribution à l'étude du parasitisme du châtaignier par le Phytophthora cinnamomi Rands. Annales de Phytopatalogie 6, 83–90.
8. Clarke, A. E. & Stone, B. A., 1962. β-1,3-glucan hydrolysis from the grape vine (Vitis vinifera) and other plants. Phytochemistry 1, 175–188.
9. Davison, E. M., 1968. Cytochemistry and ultrastructure of hyphae and haustoria of Peronospora parasitica (Pers. ex Fr.) Fr. Annals of Botany 32, 613–621.
10. Dietrich, S. M. C. & Valio, I. F.M., 1973. Effect of coumarin and its derivatives on the growth of Pythium and other fungi. Transactions of the British mycological Society 61, 461–469.
11. Heath, M. C., 1971. Haustorial sheath formation in cowpea leaves immune to rust infection. Phytopathology 61, 383–388.
12. Hohl, H. R., 1975. Levels of nutritional complexity in Phytophthora: lipids, nitrogen sources and growth factors. Phytopathologische Zeitschrift 84, 18–33.
13. Hohl, H. R. & Stössel, P., 1976. Host-parasite interfaces in a resistant and a susceptible cultivar of Solanum tuberosum inoculated with Phytophthora infestans: tuber tissue. Canadian Journal of Botany 54, 900–912.
14. Holten, V. Z. & Bartnicki-Garcia, S., 1972. Intracellular β-glucanase activity of Phytophthora palmivora. Biochimica Biophysica Acta 276, 221–227.
15. Horikawa, T., Tomiyama, K. & Doke, N., 1976. Accumulation and transformation of rishitin and lubimin in potato tuber tissue infected by an incompatible race of Phytophthora infestans. Phytopathology 66, 1186–1191.
16. Kelley, W. D., 1975. Physiological differences among isolates of Phytophthora cinnamomi. Canadian Journal of Microbiology 21, 1548–1552.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J., 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265–275.
18. McIntyre, J. L. & Hankin, L., 1978. An examination of enzyme production by Phytophthora spp. on solid and liquid media. Canadian Journal of Microbiology 24, 75–78.
19. Meyer, R., Parish, R. W. & Hohl, H. R., 1976. Hyphal tip growth in Phytophthora. Archives of Microbiology 110, 215–224.
20. Reuveni, M. & Cohen, Y., 1978. Growth retardation and changes in phenolic compounds, with special reference to scopoletin, in mildewed and ethylene-treated tobacco plants. Physiological Plant Pathology 12, 179–189.
21. Stoessl, A., Unwin, C. H. & Ward, E. W. B., 1972. Post-infectional inhibitors from plants. I. Capsidiol, an antifungal compound from Capsicum frutescens. Phytopathologische Zeitschrift 74, 141–152.
22. Tokunaga, J. & Bartnicki-Garcia, S., 1971. Structure and differentiation of the cell wall of Phytophthora palmivora: cyst, hyphae and sporangia. Archiv für Mikrobiologie 79, 293–310.