

## Production of extracellular enzymes by germinating cysts of *Phytophthora infestans*

MOREAU, R. A., and T. S. SEIBLES. 1985. Production of extracellular enzymes by germinating cysts of *Phytophthora infestans*. *Can. J. Bot.* **63**: 1811–1816.

Cysts of *Phytophthora infestans* were prepared and allowed to germinate in water for 0 to 20 h. The activities of 11 different types of hydrolytic enzymes were detected in the extracellular germination medium. A time-course study revealed that most of the enzyme activities increased very little during germination. However, esterase activity increased 45-fold during germination. The rate of appearance of esterase activity closely paralleled the rate of germ tube growth. The intracellular levels of esterase activity were low throughout germination. These observations suggest that esterase is secreted during germination. Cysts also were allowed to germinate in the presence of various potential metabolic inhibitors and their effect on the appearance of esterase activity and on germ tube growth was measured. With each compound that inhibited the rate of germ tube growth, there was a nearly proportionate inhibition in the rate of appearance of extracellular esterase.

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Des kystes de *Phytophthora infestans* préalablement préparés ont été mis à germer dans de l'eau pendant des durées de 0 à 20 h. Les activités de 11 types différents d'enzymes hydrolytiques ont été décelées dans le milieu de germination extracellulaire. Une étude chronoséquentielle a révélé que la plupart des activités enzymatiques n'augmentent que très peu pendant la germination. Cependant, l'activité estérasique augmente d'un facteur de 45 au cours de la germination. Le taux d'apparition de l'activité estérasique suit de près le taux de croissance du tube germinatif. L'activité estérasique intracellulaire demeure faible tout au long de la germination. Ces observations suggèrent que l'estérase est sécrétée pendant la germination. Dans une autre expérience, des kystes ont été mis à germer en présence de divers inhibiteurs métaboliques potentiels et leur effet sur la croissance des tubes germinatifs a été mesurée. Tous les composés qui inhibent le taux d'élongation des tubes germinatifs entraînent une inhibition presque proportionnelle dans le taux d'apparition de l'estérase extracellulaire.

[Traduit par le journal]

### Introduction

It has previously been reported that *Phytophthora* species secrete many types of enzymes during vegetative growth; these include pectate transeliminase (16), polygalacturonase (12,16), pectinesterase (12), cellulase (16), amylase (16), invertase (26),  $\beta$ -1,3-glucanase (9,22), galactanase (12),  $\beta$ -glucosidase (22), DNase (16), RNase (16), protease (16), acid phosphatase (17), lipase (16,18), phospholipase (18), and esterase (18). In contrast, very little is known about the secretion of enzymes during the germination of spores of any fungal species. It has been suggested that  $\beta$ -glucosidase,  $\beta$ -1,3-glucanase, and acid phosphatase are secreted during the germination of cysts of *Phytophthora palmivora* (17).

This study was undertaken to compare the types of enzymes secreted during germination of cysts of *Phytophthora infestans* with those previously reported in culture filtrates. Because the initial stages of infection by this phytopathogen occur during cyst germination (5), an understanding of the types of enzymes secreted during germination may increase our understanding of the infection process. Cysts were germinated in water and the levels of various enzymes were measured during germination. Several compounds that had previously been reported to inhibit the germination of fungal spores were also tested for their ability to inhibit the secretion of enzymes during germination.

### Material and methods

#### Preparation of intracellular and extracellular extracts

Cultures of *P. infestans* (Mont.) de Bary were obtained from W. Fry, Ithaca, New York (isolates R-OA and R-4A), and R. Goth, Beltsville, Maryland (isolates R-OV and R-1,2V). All isolates were maintained on corn seed media to help retain virulence (7). Infected corn seeds were used to inoculate lima bean agar (18) in Petri dishes. After 14 days growth at 14°C, sporangia were harvested by flushing the surface of the agar with 15 mL of distilled water and lightly

scraping with a bent glass rod. Mycelia were removed by filter sporangia preparation through two layers of cheesecloth. Spore suspensions (30-mL aliquots) were transferred to clean Petri and incubated uncovered at 14°C for 2–3 h to induce the re zoospores. Empty sporangia cases were removed by filtering tile zoospores through Whatman No. 4 filter paper. Zoospore counts were made with a counting chamber (Petroff–Hausser) so that enzyme activities could be normalized (per 10<sup>6</sup> spores). The zoospore suspensions were transferred to large test tubes (200 × 25 mm) and mixed on a vortex for 60 s to induce synchronous encystment (23). Cyst preparations were placed in clean Petri dishes and allowed to germinate at 14°C for up to 20 h. After 20 h of germination there was no visible evidence of bacterial contamination when the preparations were viewed with a 600 power objective on a phase contrast microscope. After the desired time, germination fluids were collected and centrifuged at 10 000 × g for 20 min to remove germinating cysts. Germination fluids were then assayed for enzyme activity immediately or after concentration with an ultrafiltration apparatus (Amicon<sup>1</sup> model 202 with PM-10 membranes).

Intracellular extracts were prepared by centrifuging 0-h or 20-h cyst preparations at 10 000 × g for 10 min. The pelleted spores were homogenized for 30 s in a Bead Beater cell disrupter (Biospec Products) with 10 g glass beads (0.2–0.5 mm) and 30 mL of buffer containing 0.3 M sucrose, 0.1 M HEPES, pH 7.5, and 2 mM EDTA. After the beads had settled, the cell homogenate was assayed directly for esterase activity.

#### Polyacrylamide gel electrophoresis

Electrophoresis of samples was carried out in polyacrylamide gel cylinders according to Davis (6), but without the use of a large-pore sample gel. Samples were dissolved in 62 mM Tris–HCl (pH 6.8) containing 2.5% sucrose. Electrophoretic separations were accomplished at 5 mA per gel cylinder. Following electrophoresis, gels were

<sup>1</sup>Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE 1. Occurrence of hydrolytic enzyme activities in germination fluids of *P. infestans*, isolate R-1,2V, after 20 h of germination

Enzyme	Substrate	pH	Enzyme activity, nmol · min <sup>-1</sup> · 10 <sup>-6</sup> spores
Esterase	PNP butyrate	5	332
		6	411
		7	455
		8	407
		9	— <sup>a</sup>
	PNP palmitate	5	42.0
		6	51.3
		7	46.1
		8	44.4
β-1,3-Glucanase	Laminarin	5	1.47
		7	1.73
		8	1.69
β-Glucosidase	PNP glucose	5	1.36
		7	1.24
		9	0.080
Cellulase	Carboxymethyl cellulose	5	0.665
		7	0.843
		8	0.747
Phospholipase	Phosphatidylcholine	5	0.050
		7	0.110
		9	0.610
Galactanase	Potato pectin	5	0.483
		7	0.172
		8	0.143
Lipase	Triolein	9	0.138
Acid phosphatase	PNP phosphate	5	0.130
β-Galactosidase	PNP-β-galactose	5	0.029
Alkaline phosphatase	PNP phosphate	8	0.020
β-Galacturonase	PNP-β-galacturonate	5	0.0090
Polygalacturonase	Pectin	5	ND <sup>b</sup>
	Polygalacturonic acid	5	ND
	Pectin	5	ND
Polygalacturonic acid lyase	Pectin	5	ND
	Polygalacturonic acid	5	ND
Protease	Azoalbumin	5	ND
		7	ND
		9	ND
Wax ester hydrolase	Stearyl stearate	5	ND
Sterol ester hydrolase	Cholesterol oleate	5	ND

<sup>a</sup>Background rate of nonenzymatic hydrolysis was too high to measure enzyme-dependent hydrolysis.

<sup>b</sup>ND, not detected.

either immersed in 0.25% Coomassie brilliant blue R-250 dye dissolved in methanol – water – acetic acid (4.9:4.9:1) for approximately 1 h, or immersed in a stain for esterase activity which consisted of 1 mL of 10 mM 4-methylumbelliferyl butyrate (4-MU butyrate) in ethylene glycol monomethyl ether and 50 mL of 50 mM potassium phosphate buffer, pH 8. Coomassie blue stained gels were destained with several changes of ethanol – acetic acid – water (3:1:10). Esterase activity was detected as a blue fluorescent band when the gels were viewed under an ultraviolet lamp (366 nm).

#### Enzyme assays

All enzyme assays were conducted with the following buffer systems: citrate – sodium citrate for pH 5.0, potassium phosphate for pH 6, 7, and 8, and glycine – sodium hydroxide for pH 9.0.

Esterase was assayed as previously described (18). The reaction mixture (1 mL) contained 0.1 M potassium phosphate buffer, pH 8.0, 0.1% Triton X-100, 2 mM *p*-nitrophenyl butyrate (or 0.2 mM *p*-nitrophenyl palmitate), and 5 to 25 μL germination medium (molar extinction coefficient = 15.1 cm<sup>2</sup>/μmol). When other buffers were tested (Table 1) the pH was adjusted to 8.0 immediately before the absorbance was measured.

Phospholipase and lipase activities were assayed with <sup>14</sup>C-labelled substrates as previously described (18). The reaction mixture contained 100 μL phosphatidylcholine-dipalmitoyl (or triolein), 0.01% Triton X-100, 0.1 M glycine – sodium hydroxide (pH 9.0), and 25 to 100 μL germination fluid.

Sterol ester hydrolase and wax ester hydrolase activities were assayed as previously described (18). The reaction mixture contained 0.3 mM wax ester (stearyl stearate) or 0.3 mM sterol ester (cholesterol oleate), 0.1 M citrate – sodium hydroxide (pH 5.0), 0.1% Triton X-100, and 100 μL concentrated (30×) germination fluid. The release of fatty acids was measured by a sensitive spectrophotometric assay (20). Palmitic acid was used as a standard.

Cellulase, β-1,3-glucanase, galactanase, and polygalacturonase activities were assayed by measuring the rate of release of reducing sugars (21). The reaction mixture (1 mL) contained 5 mg substrate, 0.1 M buffer (see Tables 1 and 2) and 100 μL concentrated (30×) germination fluid.

β-Glucosidase, β-galactosidase, β-galacturonase, acid phosphatase, and alkaline phosphatase were measured with *p*-nitrophenyl (PNP) derivatives. The reaction mixture (1 mL) contained 2 mM substrate, 0.1 M buffer (see Tables 1 and 2), and germination fluid. The

TABLE 2. Change in enzyme levels in germination fluids during 20 h of germination by cysts of *P. infestans* isolate R-1,2V

Enzyme <sup>a</sup>	pH	Enzyme activity, nmol · min <sup>-1</sup> · 10 <sup>-6</sup> spores		% increase
		0 h	20 h	
Esterase (PNP butyrate)	8	9.0	407	4522
β-1,3-Glucanase	7	1.59	1.73	8.8
β-Glucosidase	5	1.25	1.36	9.7
Cellulase	7	0.789	0.843	7.0
Phospholipase	9	0.590	0.610	3.4
Galactanase	5	0.330	0.483	46.4
Lipase	9	0.127	0.138	9.1
Acid phosphatase	5	0.078	0.130	66.3
β-Galactosidase	5	0.020	0.029	45.0
Alkaline phosphatase	8	0.019	0.020	8.6
β-Galacturonase	5	0.0060	0.0090	50

<sup>a</sup>The concentrations of total extracellular protein at 0 and 20 h were 8.0 and 12.9  $\mu\text{g} \cdot 10^{-6}$  spores, respectively. The pH values of the 0- and 20-h germination fluids were 6.42 and 6.97, respectively.

reactions were stopped by adding 2.5 mL of 0.5 N NaOH. The product, *p*-nitrophenol, was measured at 405 nm.

Polygalacturonic acid lyase was assayed by measuring the change in absorbance at 240 nm with polygalacturonic acid as substrate and at 235 nm with citrus pectin as substrate. The reaction mixture (1 mL) contained 5 mg substrate, 0.1 M buffer (see Table 1), and 100  $\mu\text{L}$  concentrated (30 $\times$ ) germination fluid.

Protease was assayed with azoalbumin as previously described (24). The reaction mixture (1 mL) contained 150  $\mu\text{g}$  azoalbumin, 100 mM buffer (see Table 1), 10 mM  $\text{CaCl}_2$ , and 100  $\mu\text{L}$  concentrated (30 $\times$ ) germination fluid.

#### Protein assay

Samples of germination fluid were mixed with an equal volume of 10% trichloroacetic acid and incubated at 0°C for at least 1 h. The mixture was centrifuged at 5000  $\times g$  for 10 min. The pellet was resuspended in 0.1 N NaOH and protein was assayed by the Lowry procedure (15).

## Results

Using the procedures described in the Material and methods section, >90% of the sporangia released zoospores and >90% of the cysts germinated. The addition of exogenous  $\text{Ca}^{2+}$  (0.1 mM) during the harvest of sporangia had no effect on the subsequent rate of cyst germination or enzyme levels. Although  $\text{Ca}^{2+}$  is required for germination (2) (and perhaps for secretion), adequate levels are probably carried into the germination medium from the bean agar or the mycelia during the harvest of sporangia. It is probable that small quantities of other compounds were also leached from the bean agar. If so, these compounds could potentially serve to induce or repress the production of some enzymes *in vitro*. Many attempts were made to circumvent this potential problem by rinsing the sporangia, zoospores, or cytozoospores and replacing the fluid with a defined medium (various concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , buffer, and glucose). In each case many spores were lysed and there was a very poor germination rate. Even the volume of water that was added to each Petri dish to harvest sporangia was crucial. Only with 10–20 mL/culture did we obtain consistently good release of zoospores and germination of cysts.

After 20 h of germination the activities of many diverse

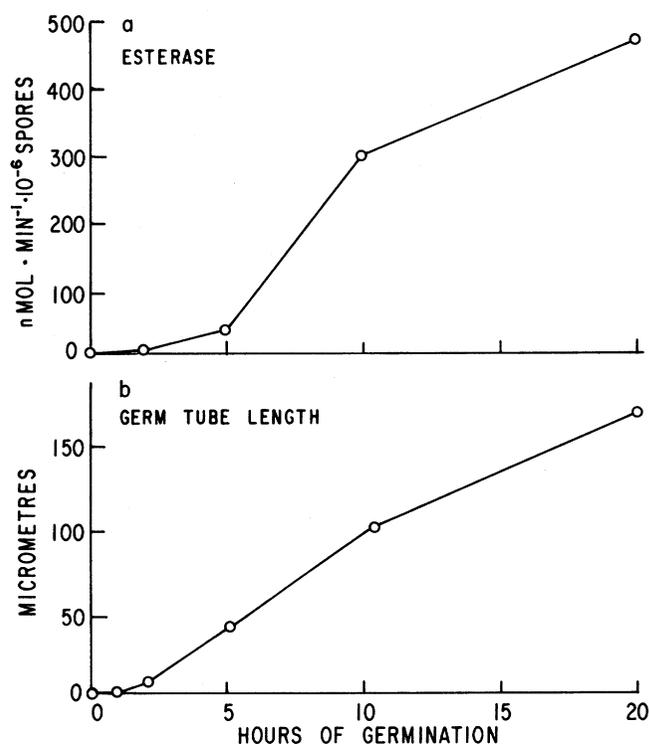


FIG. 1. (a) Time-course study of esterase activity (PNP-butyratase hydrolase) in germination fluid ( $n = 100$ ). (b) Time-course study of average germ tube length of germinating cysts ( $n = 100$ ).

enzymes were measured in the cell-free germination medium (Table 1). The six highest enzyme activities were each assayed at several pH levels to determine the pH optimum of each for the subsequent experiments. The most substantial enzyme activity, by far, was an esterase activity which hydrolyzed the two substrates shown in Table 1. The next most abundant activities in the germination fluids were  $\beta$ -1,3-glucanase,  $\beta$ -glucosidase, and cellulase. A B-type phospholipase activity which we have recently described (18) was next, followed by a galactanase activity which hydrolyzes galactose residues from potato pectin as previously described (12). Lower levels of lipase, acid phosphatase,  $\beta$ -galactosidase, alkaline phosphatase, and  $\beta$ -galacturonase were also detected. The activities of polygalacturonase, polygalacturonic acid lyase, protease, wax ester hydrolase, and sterol ester hydrolase were not detected by our assay techniques. This experiment and all others were performed at least three times, and the values reported are the averages of at least three replicates.

To determine which enzyme activities might be actively secreted during germination of cysts, assays were run immediately after encystment (0 h) and the results are compared with those obtained after 20 h of germination (Table 2). Of the 11 enzyme activities that were measured, only the esterase activity exhibited a substantial increase (45-fold) during germination. Although the other enzymes activities all increased during germination, the increases were very modest in comparison to that of the esterase. A time-course study of the appearance of esterase activity during germination revealed that the rate of appearance of esterase closely paralleled the rate of germ tube growth (Fig. 1). Enzyme samples (50  $\mu\text{L}$  of 30 $\times$  concentrate) from the same time course also were examined by polyacrylamide gel electrophoresis (Fig. 2). Very few bands were present at 0 h. During germination four new major

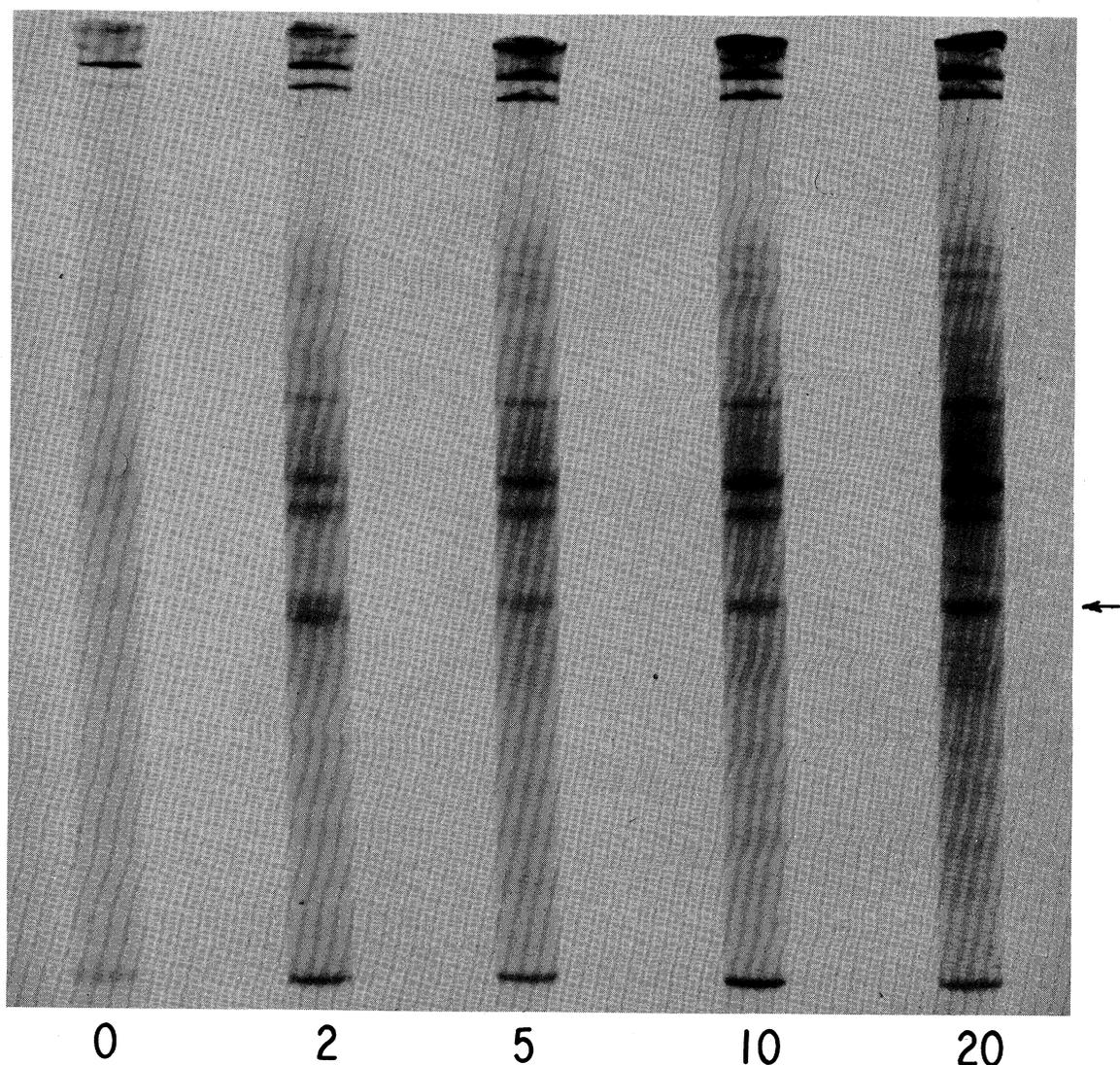


FIG. 2. Time-course study of proteins in germination medium as separated by polyacrylamide gel electrophoresis. Numbers represent hours of germination. Arrow indicates esterase activity stain (region of fluorescence when gels were immersed in 4-MU butyrate).

TABLE 3. Comparison of levels of intracellular and extracellular esterase from ungerminated (0 h) and germinated (20 h) cysts of *P. infestans* isolate R-1,2V

	Esterase activity (PNP-butyrate hydrolase), $\text{nmol} \cdot \text{min}^{-1} \cdot 10^{-6}$ spores	
	0 h	20 h
Extracellular	9.0	407.3
Intracellular	1.8	2.6
Intracellular + extracellular (1:1 mixture)	11.6	416.1

bands and several minor bands appeared. When the gels were dipped in 4-methylumbelliferyl butyrate as an esterase activity stain, one of the major bands fluoresced as noted in Fig. 2. The only other activity stain that was tried, 4-methylumbelliferyl- $\beta$ -glucose, caused a broad band of fluorescence near the top of the gel; this band did not contain enough protein to stain with Coomassie blue. We do not know the origin of the protein

band that appears only at 2 h, immediately below the esterase band.

Because of the dramatic change in the levels of extracellular esterase activity during germination, an experiment was performed to examine the levels of intracellular esterase (Table 3). At 0 h of germination the intracellular esterase activity was about fivefold lower than the extracellular activity. During germination there was a small increase in intracellular esterase activity and a 45-fold increase in extracellular activity. These results indicate that the extracellular esterase is apparently secreted during germination and is not derived from lysis. In cases where extensive lysis was observed (as in the KCN and thimerosal treatments; discussed later), esterase activity was undetectable (even less than pregermination levels), which suggests that proteases were released. When intracellular and extracellular extracts were combined (1:1 mixture), there was no evidence of the presence of activators or inhibitors in either extract (Table 3).

The experiments that have been described so far were all performed with one isolate of *P. infestans*, R-1,2V. An experiment was then designed to verify whether the patterns of

TABLE 4. Comparison of enzyme activities in germination fluids of four different isolates of *P. infestans* after 20 h of germination

Isolate	Physiologic race	Enzyme activity	
		(PNP-butyrate hydrolase), nmol · min <sup>-1</sup> · 10 <sup>-6</sup> spores	β-Glucosidase activity, nmol · min <sup>-1</sup> · 10 <sup>-6</sup> spores
R-1,2V	1,2	538	3.16
R-4 A	4	1373	1.11
R-0 A	0	283	2.40
R-OV	0	285	2.84

NOTE: Esterase was assayed at pH 8.0 and β-glucosidase was assayed at pH 5.0.

TABLE 5. Effects of various potential inhibitors on germ tube length and extracellular esterase activity after 20 h of germination (*P. infestans* isolate R-1,2V)

Treatment	Concentration	Enzyme activity	
		Average germ tube length, μm	(PNP-butyrate hydrolase), nmol · min <sup>-1</sup> · 10 <sup>-6</sup> spores
Control		160 ± 11 (100)	433 ± 31 (100)
Glucose	0.1 M	163 ± 19 (102)	637 ± 8 (147)
Cycloheximide	4 mM	0	27 ± 4 (6)
KCN	5 mM	0 <sup>a</sup>	0
Thimerosal	0.01%	0 <sup>a</sup>	0
Tunicamycin	2 μg/mL	48 ± 9 (30)	136 ± 12 (32)
	20 μg/mL	40 ± 11 (25)	71 ± 6 (16)
Colchicine	2 mM	160 ± 21 (100)	554 ± 34 (128)
Nocodazole <sup>b</sup>	10 μM	120 ± 15 (75)	315 ± 19 (73)
	100 μM	107 ± 33 (67)	294 ± 17 (68)
Cytochalasin B <sup>c</sup>	100 μM	166 ± 23 (101)	524 ± 21 (121)
Cerulenin <sup>c</sup>	50 μg/mL	44 ± 13 (28)	87 ± 9 (20)
Trifluoperazine	101 μM	110 ± 15 (69)	165 ± 29 (38)
	50 μM	0	0
Chlorpromazine	10 μM	107 ± 22 (67)	139 ± 26 (32)
	50 μM	0	0
Dibucaine	50 μM	69 ± 19 (43)	360 ± 3 (83)

NOTE: Numbers in parentheses are relative activities or relative lengths (control = 100).

<sup>a</sup>All cysts were lysed after 20 h.

<sup>b</sup>Dimethyl sulfoxide (0.5%) control.

<sup>c</sup>Ethanol (1%) control.

enzymatic activities that we observed with one isolate were repeatable with three other isolates of the same species (Table 4). After 20 h, the levels of esterase and β-glucosidase activities in the germination fluids from all four isolates were not identical but were within the same order of magnitude.

An experiment was designed to measure the effect of potential inhibitors on cyst germination and the secretion of esterase (Table 5). We were especially interested in determining whether there were any compounds that would inhibit secretion but not effect germ tube elongation or vice versa. Glucose had no effect on germ tube length but stimulated esterase activity by 47%. This observation indicates that some processes in germinating cysts may not be under catabolite repression. Cycloheximide, cyanide, and thimerosal (a mercuric antimicrobial compound) all inhibited germination and secretion of esterase. In a previous report (4), 4 mM cycloheximide also caused complete inhibition of spore germination. With cycloheximide all of the cysts remained intact after 20 h; however, with both KCN and thimerosal all of the cysts were lysed after 20 h. The absence of any detectable esterase activity (less than the pre-germination level in Table 2) in the KCN and thimerosal treatments suggests that proteases were probably released during cell lysis. Tunicamycin is an inhibitor of glycoprotein synthesis (14). At a concentration of 2 μg/mL the rate of germ tube

growth was reduced by 70% and esterase activity was 68% lower. At 20 μg/mL, germ tube length was diminished by 75% and esterase levels by 84%. These data suggest that both germ tube elongation and the synthesis and secretion of esterase require the synthesis of new glycoproteins. Colchicine had no effect on germ tube elongation and actually caused a significant (28%) stimulation of esterase levels. Colchicine is a potent antimicrotubule agent in most eukaryotes, but several other fungal species are unaffected by colchicine (8). Nocodazole has been found to be a good antimicrotubule agent in most fungi (8), and in our hands it inhibited both germ tube growth and esterase activity by 25–33%. Cytochalasin B, an antimicrofilament agent, was shown to cause unusual swelling in the germ tubes of cowpea rust fungus (8). Germ tube length was unaffected by cytochalasin B, and the appearance of esterase activity was slightly stimulated. Cerulenin, an inhibitor of fatty acid synthetase, was reported to inhibit germination of *Botryodiplodia* (1) and *Rhizopus* (19) spores, and inhibited secretion of glucosyl transferase by *Streptococcus salivarius* (11). With *P. infestans* spores germ tube length and the appearance of esterase activity were equally inhibited (72–80%). It was recently reported that trifluoperazine, a calmodulin antagonist, reduced the viability of *P. cinnamomi* zoospores (10). Trifluoperazine (10 mM) and two other calmodulin antagonists

(chlorpromazine and dibucaine) each caused a significant inhibition of germ tube growth and appearance of esterase activity. In conclusion, this preliminary study indicates that each of the compounds that inhibited germination also caused a nearly proportionate inhibition in the rate of appearance of extracellular esterase.

### Discussion

The results of this study indicate that the types and quantities of enzymes secreted during germination may be very different from those secreted during vegetative growth. Among the many enzyme activities that had been detected in culture filtrates of *P. infestans*, 11 were observed in the postgermination medium, but only 1, the esterase, appeared to be actively secreted during germination. In previous studies with other fungal species, germinating spores were shown to secrete pectinases (25) and cutinases (13). We are now in the process of purifying the esterase and assessing its ability to hydrolyze natural substrates (i.e., cutin, waxes, and lipids). Our preliminary results indicate that the esterase activity corresponds to a single protein with a molecular weight of about 26 000.

The small increases (7–66%) in the levels of other enzymes during germination can be interpreted in other ways. Our *in vitro* germination system may contain enzymatic repressors or enzyme inhibitors which were leached from the bean agar during the harvest of sporangia. In contrast, some of these enzymes may only be secreted in the presence of inducers or activators. It would be very interesting to learn which events during spore germination are constitutive and which are regulated by inducers and other environmental stimuli. Among the extracellular enzyme activities that exhibited little change during germination, those of  $\beta$ -1,3-glucanase (9,17),  $\beta$ -glucosidase (17), and galactanase (12) have previously been reported in spores of other *Phytophthora* species, but this is the first time that the levels of these enzymes have been monitored during cyst germination. Finally, the small increase in the activities of these enzymes during germination may be caused by molecular sieving within the fungal cell wall as previously described (3). A possible reason why the esterase activity increased so dramatically during germination may be because it has a low molecular weight, allowing it to easily traverse the cell wall.

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