APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1994, p. 489–495
0099-2240/94/$04.00+0

Vol. 60, No. 2

Production of Chitinases and β-1,3-Glucanases by *Stachybotrys elegans*, a Mycoparasite of *Rhizoctonia solani*

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Received 30 August 1993/Accepted 10 November 1993

The in vitro production of chitinases and β-1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*, was examined under various culture conditions, such as carbon and nitrogen sources, pH, and incubation period. Production of both enzymes was influenced by the carbon source incorporated into the medium and was stimulated by acidic pH and $NaNO_3$. The activity of both enzymes was very low in culture filtrates from cells grown on glucose and sucrose compared with that detected on chitin (for chitinases) and cell wall fragments (for β-1,3-glucanases). Protein electrophoresis revealed that, depending on the carbon source used, different isoforms of chitinases and β-1,3-glucanases were detected. *S. elegans* culture filtrates, possessing β-1,3-glucanase and chitinase activities, were capable of degrading *R. solani* mycelium.

The rhizoctonia disease of potatoes known as blackscurf and stem canker is a problem of worldwide occurrence. It is widespread in North America and endemic to many regions of Canada, including the province of Québec (2). This disease is mainly caused by isolates of *Rhizoctonia solani* Kühn belonging to anastomosis group 3 (20), and it is characterized by the presence of sclerotia on tubers, which causes irreversible damage on the underground parts of the growing potato plant (2). Since varieties of potatoes resistant to *R. solani* diseases do not exist and cultural methods are not always efficient, an increasing amount of research is being directed toward the biological control of this pathogen, using antagonistic microorganisms as an alternative to chemicals. Antagonism may operate by antibiosis, competition, predation, or parasitism (21). Parasitism involves the production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi (12). Chitinases and β-1,3-glucanases are considered key hydrolytic enzymes in the lysis of cell walls of higher fungi (7, 17). These enzymes have been shown to be produced by several fungi and bacteria and may be an important factor in biological control (1, 11, 19).

Stachybotrys elegans (Pidopl.) W. Gams, isolated from the soil, was shown to have a strong antagonistic activity against *R. solani* in in vitro studies (4, 27). Benyagoub and Jabaji-Hare (4) demonstrated that parasitism is the main mechanism involved in the interaction between *S. elegans* and *R. solani*. Partial degradation of *R. solani* mycelial and sclerotial cell walls at the site of interaction with *S. elegans* was confirmed in ultrastructural studies and strongly suggests the production of hydrolytic enzymes such as chitinases and β-1,3-glucanases.

With a view to establish that the mechanism in the process of parasitism of *R. solani* by *S. elegans* involves the release of lytic enzymes by the latter, the objectives of this study were (i) to determine the physiological conditions which stimulate the in vitro production of chitinases and β-1,3-glucanases and (ii) to study the effect of culture filtrates possessing hydrolytic activity on *R. solani* mycelium.

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MATERIALS AND METHODS

Organisms and cultivation. The mycoparasite *S. elegans* was isolated from soil in Izmir, Turkey, and the pathogen *R. solani* (anastomosis group 3) was isolated from infested field potato tubers with sclerotia. *S. elegans* was grown on sterile oat seeds for 10 days by the method of Escande and Echandi (13), while *R. solani* was maintained on potato dextrose agar (Difco Laboratories, Detroit, Mich.) slants. The oat seeds and agar slants were stored at 4°C and served as stock cultures. Inoculum for enzymatic studies consisted of potato dextrose agar disks of 10-day-old mycelium of *S. elegans* that was grown from colonized oat kernels.

Preparation of *R. solani* cell wall fragments. *R. solani* cell wall fragments were prepared by the method of Chet et al. (8) with some modifications. Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth (Difco) were incubated with agar disks of actively growing mycelium of *R. solani* that was previously grown on potato dextrose agar for 7 days. The inoculated flasks were incubated at 24°C for 7 days and shaken at 110 rpm. The mycelium was then collected by filtration through Whatman no. 1 filter paper, washed with sterile water, homogenized in a Waring blender, and treated in an ultrasonic disintegrator (sonic dismembrator; Quigley-Rochester Inc., Rochester, N.Y.) for 10 min. Cell wall fragments were centrifuged twice (160 and 16,000 × *g*) for 10-min periods and then washed with sterile water until no residual glucose and proteins could be detected in the supernatant. Glucose and protein concentrations were determined with the glucose oxidase reagent (Sigma Chemical Co., St. Louis, Mo.) and by the Bradford (5) method, respectively. Purified cell wall fragments were freeze-dried, ground into powder with a mortar and pestle, and autoclaved at 121°C for 15 min. Freeze-dried, powdered *R. solani* mycelium was prepared by the same protocol as cell wall fragments, except the ultrasonic disintegrator treatment was omitted.

Conditions for enzyme production. Growth was carried out on minimal synthetic medium (MSM) containing the following (in grams per liter): $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl,

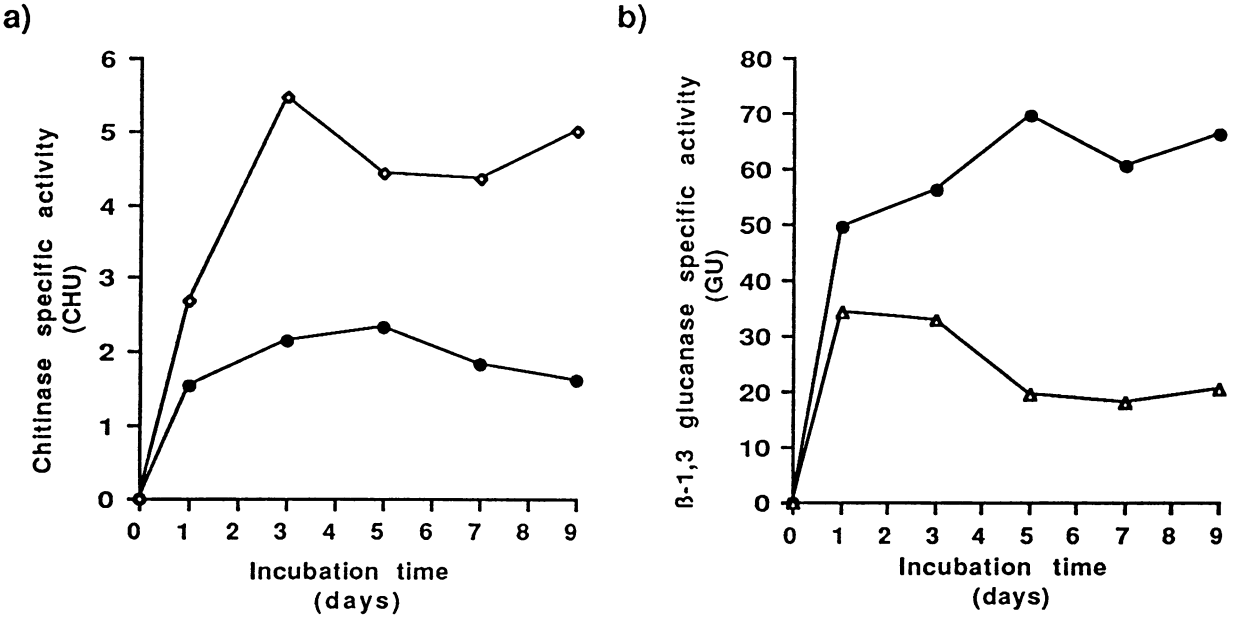

FIG. 1. Time course of (a) chitinase and (b) β-1,3-glucanase production by *S. elegans* on MSM (no carbon source) (●) and on medium containing chitin (◇) or laminarin (△) as carbon source (1 mg ml^{-1}), respectively. Chitinase and β-1,3-glucanase specific activities are expressed as micromoles of *N*-acetylglucosamine (CHU) and as micromoles of glucose (GU) per milligram of protein per hour, respectively. Each value represents the mean of four separate determinations.

0.2; $FeSO_4 \cdot 7H_2O$, 0.002; $MnSO_4$, 0.002; and $ZnSO_4$, 0.002; the medium was supplemented with the appropriate carbon and nitrogen sources (1 mg ml^{-1}). Unless stated otherwise, the pH of the medium was adjusted with KOH and HCl to 6.3 and the nitrogen source was NH_4NO_3. Flasks (250 ml) containing 50 ml of culture media were incubated with shaking (110 rpm) at 24°C for various time periods. Culture filtrate from each flask was collected by filtration through Whatman no. 1 filter paper and freeze-dried.

Time course of chitinase and β-1,3-glucanase production. Growth was carried out on either chitin-containing medium or MSM (no carbon source added, but containing NH_4NO_3 as nitrogen source) to determine chitinase activity. To assess β-1,3-glucanase activity, laminarin-containing medium or MSM was used. Quadruplicate flasks were removed after 1, 3, 5, 7, and 9 days of growth for enzyme assays.

Effect of carbon and nitrogen sources on chitinase and β-1,3-glucanase production. Growth was carried out on medium containing either no carbon source (MSM), glucose (Sigma), sucrose (Fisher Scientific Co., Montreal, Québec, Canada), *N*-acetyl-D-glucosamine (Sigma), *R. solani* cell wall fragments, purified chitin from crab shells (Sigma), or purified laminarin from *Laminaria digitata* (Sigma). The carbon source concentration used was 1 mg ml^{-1}. Triplicate flasks were removed after 5 days of growth for enzyme assays. The effect of different nitrogen sources on chitinase and β-1,3-glucanase production was tested in MSM containing chitin and laminarin, respectively. The nitrogen sources applied at 1 mg ml^{-1} were NH_4NO_3 (25 mM nitrogen; GIBCO-BRL Co., Burlington, Ontario, Canada), $NaNO_3$ (11.8 mM nitrogen; Fisher), asparagine (15.1 mM nitrogen; Sigma), and ammonium tartrate (10.9 mM nitrogen; BDH Chemical Co., Montreal, Québec, Canada). Quadruplicate flasks were removed after 5 days of growth for enzyme assays.

Effect of initial pH. Chitin- and laminarin-containing media (1 mg ml^{-1}) were adjusted with KOH and HCl to pH of 4, 5, 6, 7, 8, and 9. Triplicate flasks were removed after 8 h and 5 days of growth to assess β-1,3-glucanase and chitinase activities, respectively.

Enzyme assays. β-1,3-Glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) activities were estimated, with slight modifications, according to the method of Elad et al. (11). β-1,3-Glucanase activity was assayed by monitoring the release of free glucose, using glucose oxidase reagent (Sigma) according to the manufacturer's recommendations. The culture filtrates were rehydrated with 0.1 M citrate buffer, pH 4.7. The reaction mixture, which contained 2 ml of rehydrated culture filtrate and 1.6 mg of soluble laminarin, was incubated at 40°C for 1 h, and the reaction was stopped by boiling. β-1,3-Glucanase specific activity (GU) was expressed as micromoles of glucose per milligram of protein per hour at 40°C. Chitinase activity was assayed by monitoring the release of *N*-acetylglucosamine according to the method of Reissig et al. (23). The culture filtrates were rehydrated with 0.2 M phosphate buffer, pH 5.6, and the reaction mixture contained 2 ml of rehydrated culture filtrate and 10 mg of chitin. The reaction mixture was incubated at 37°C for 2 h, and the reaction was stopped by boiling. Residual chitin was removed by low-speed centrifugation. Chitinase specific activity (CHU) was expressed as micromoles of *N*-acetylglucosamine per milligram of protein per hour at 37°C. The protein concentration of culture filtrates was determined by the method of Bradford (5), using bovine serum albumin (Sigma) as the standard.

PAGE. To detect the isoforms of chitinase and β-1,3-glucanase, polyacrylamide gel electrophoresis (PAGE), under native conditions, was performed at pH 8.9 according to the method of Davis (9), using 10% (wt/vol) polyacrylamide resolving gel and 5% (wt/vol) polyacrylamide stacking gel. The resolving gel for detection of chitinase activity contained 0.01% (wt/vol) glycol chitin (26), whereas that for the detection of β-1,3-glucanase activity contained 0.6 mg of alkali-soluble *Saccharomyces cerevisiae* β-1,3-glucan ml^{-1} (14). Electro-

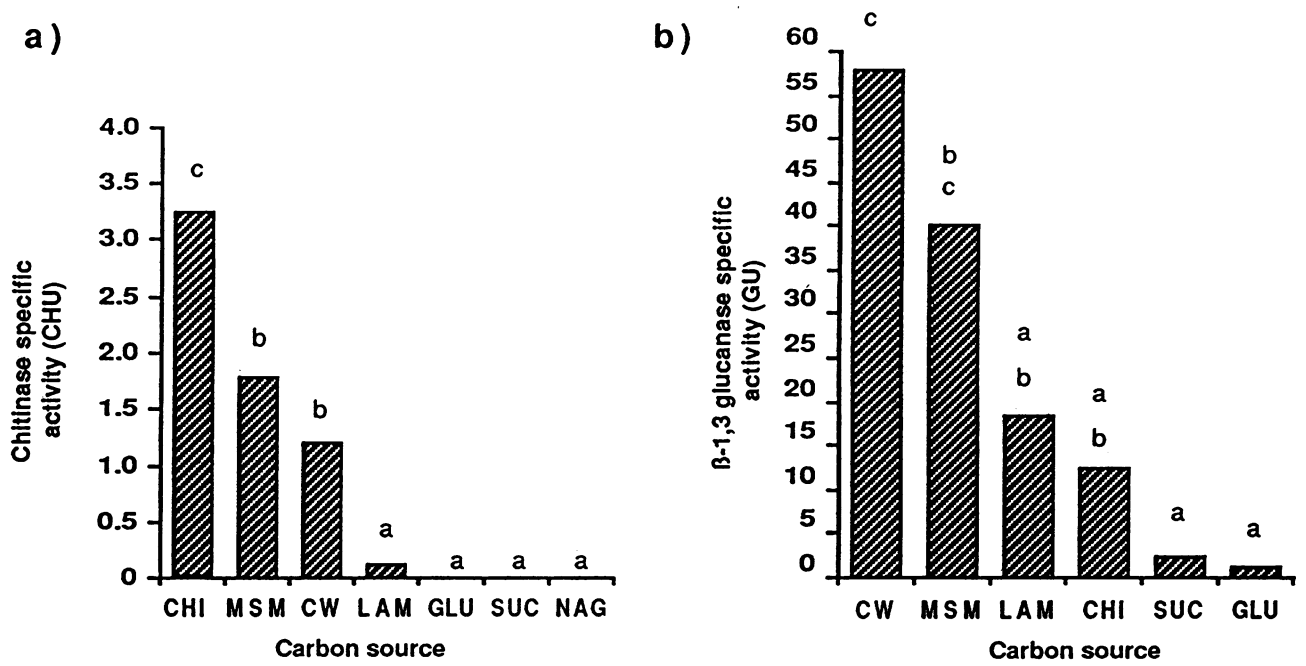

FIG. 2. Effect of carbon sources on (a) chitinase and (b) β-1,3-glucanase production after 5 days of incubation. Chitinase and β-1,3-glucanase specific activities are expressed as micromoles of *N*-acetylglucosamine (CHU) and micromoles of glucose (GU) per milligram of protein per hour, respectively. Each value represents the mean of three separate determinations. Values followed by the same letters are not significantly different (Duncan; $P \leq 0.05$). Carbon sources were as follows (1 mg ml^{-1}): chitin (CHI); *R. solani* cell wall fragments (CW); laminarin (LAM); glucose (GLU); sucrose (SUC); *N*-acetylglucosamine (NAG); and minimal synthetic medium with no carbon source (MSM).

phoresis was performed at room temperature at 25 mA for 120 (chitinase activity) or 105 (β-1,3-glucanase activity) min, and a minimum of two electrophoretic runs was performed for each enzyme. Detection of chitinase activity after PAGE was performed by incubating the gel for 2 h at 37°C in 50 mM sodium acetate buffer (pH 5.0) and staining it with Calcofluor White M2R according to the method of Trudel and Asselin (26). Detection of β-1,3-glucanase activity after PAGE was performed by incubating the gel at 37°C in 10 mM sodium acetate buffer (pH 5.0) and staining it with aniline blue (0.025% [wt/vol]) according to the method of Grenier and Asselin (14). Tobacco and barley intercellular fluids were used as positive controls for chitinase and β-1,3-glucanase activities, respectively. All chemicals for electrophoresis were obtained from Bio-Rad.

Release of glucose from *R. solani* mycelium. To test the lytic activity of culture filtrates of *S. elegans* on *R. solani*, the mycoparasite was grown for 4 days on MSM and on the following carbon sources: chitin and *R. solani* cell wall fragments. Culture filtrates were collected by filtration, freeze-dried, and rehydrated with citrate buffer. Two milliliters of each filtrate possessing β-1,3-glucanase activity (0.6 μmol of glucose ml of culture filtrate^{-1} h^{-1}) was incubated with 10 mg of *R. solani* freeze-dried, powdered mycelium at 37°C in test tubes. Glucose concentrations were determined as described previously after 10 h of incubation. Boiled culture filtrates and citrate buffer containing no culture filtrate were used as controls. Triplicate tubes were used.

RESULTS

When *S. elegans* was grown in liquid culture on chitin or laminarin as the sole carbon source, it produced extracellular chitinases and β-1,3-glucanases, respectively (Fig. 1). Production of chitinases in medium containing chitin increased up to 5.5 CHU after 3 days of growth, after which it decreased slightly. Lower activities of chitinases (1.5 to 2.4 CHU) were detected when *S. elegans* was grown on MSM. In contrast to chitinases, production of β-1,3-glucanases was higher when *S. elegans* was grown on MSM. Maximum specific activities were obtained in laminarin-containing (35 GU) and MSM (69 GU) media after 1 and 5 days of incubation, respectively. The production of extracellular chitinases and that of β-1,3-glucanases on chitin and laminarin, respectively, were not significantly different over a 9-day period ($P \leq 0.05$). Thus, an arbitrary incubation period of 5 days was chosen to test the effect of different parameters on the production of chitinases and β-1,3-glucanases.

The production of chitinases and β-1,3-glucanases by *S. elegans* was significantly influenced by the carbon source incorporated into the medium (Fig. 2). Chitinase production was significantly higher in chitin-containing medium (3.3 CHU), with yields almost threefold higher than that on cell wall fragments (1.2 CHU). Similar amounts of chitinases, which were significantly lower than those produced on cell wall fragments, were detected on laminarin, glucose, sucrose, and *N*-acetylglucosamine. The production of β-1,3-glucanases was significantly higher in medium containing *R. solani* cell wall fragments (58.0 GU) than in medium containing laminarin, chitin, sucrose, or glucose. In medium containing no carbon source (MSM), chitinase (1.8 CHU) and β-1,3-glucanase (40.0 GU) production was significantly higher than production on most other sugars.

The secretion of β-1,3-glucanases and chitinases was also significantly influenced by the nitrogen source incorporated into the medium (Fig. 3). Production of both chitinases (4.2 CHU) and β-1,3-glucanases (41.1 GU) was significantly higher

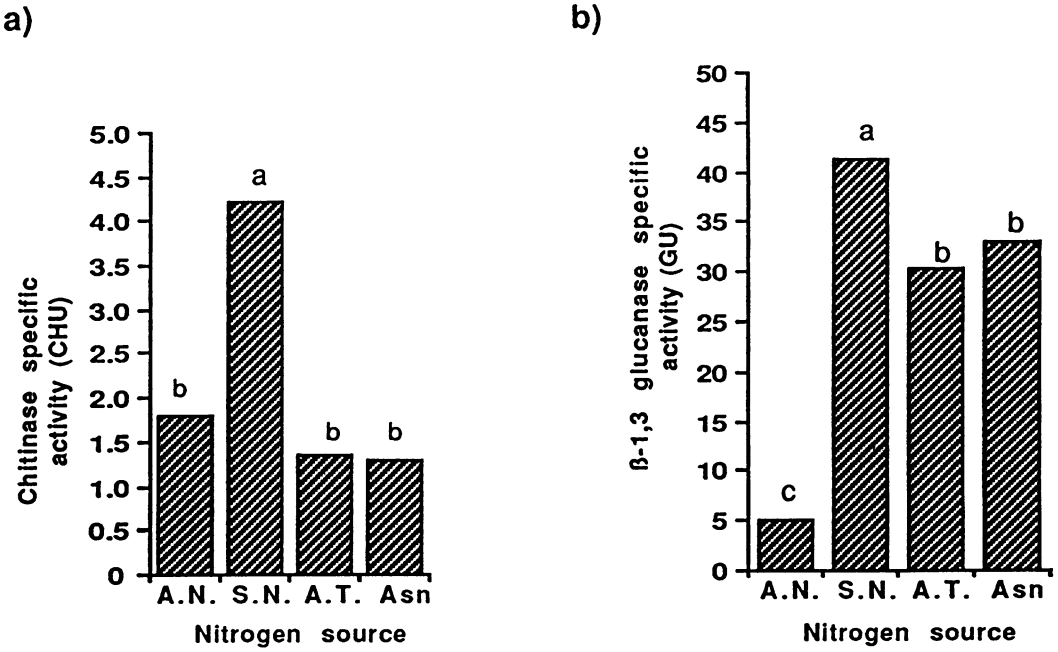

FIG. 3. Effect of nitrogen sources on (a) chitinase and (b) β-1,3-glucanase production (using chitin or laminarin [1 mg ml^{-1}] as carbon source, respectively) after 5 days of incubation. Chitinase and β-1,3-glucanase specific activities are expressed as micromoles of *N*-acetylglucosamine (CHU) and as micromoles of glucose (GU) per milligram of protein per hour, respectively. Each value represents the mean of four separate determinations. Values followed by the same letters are not significantly different (Duncan; $P \leq 0.05$). Nitrogen sources were as follows: ammonium nitrate (A.N.); sodium nitrate (S.N.); ammonium tartrate (A.T.); and asparagine (Asn).

in media containing sodium nitrate. No significant differences were observed in chitinase production when ammonium nitrate (1.8 CHU), ammonium tartrate (1.4 CHU), or asparagine (1.3 CHU) was used as the nitrogen source. β-1,3-Glucanase secretion in medium containing either ammonium tartrate (30.3 GU) or asparagine (33.1 GU) was significantly higher than that in medium containing ammonium nitrate (5.2 GU).

Chitinase production was significantly enhanced by acidic pH. Maximum production was detected at pH 5 (Fig. 4a). Compared with pH 8 (1.5 CHU) and 9 (2.0 CHU), chitinase

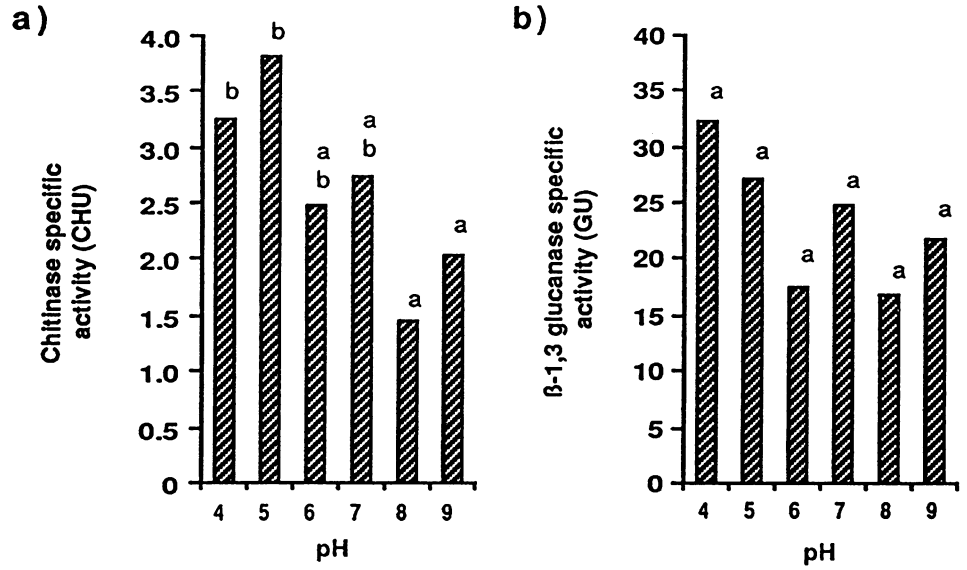

FIG. 4. Effect of initial pH on (a) chitinase and (b) β-1,3-glucanase production, using chitin (5 days of growth) or laminarin (8 h of growth) as carbon source, respectively. Chitinase and β-1,3-glucanase specific activities are expressed as micromoles of *N*-acetylglucosamine (CHU) and micromoles of glucose (GU) per milligram of protein per hour, respectively. Each value represents the mean of three separate determinations. Values followed by the same letters are not significantly different (Duncan; $P \leq 0.05$).

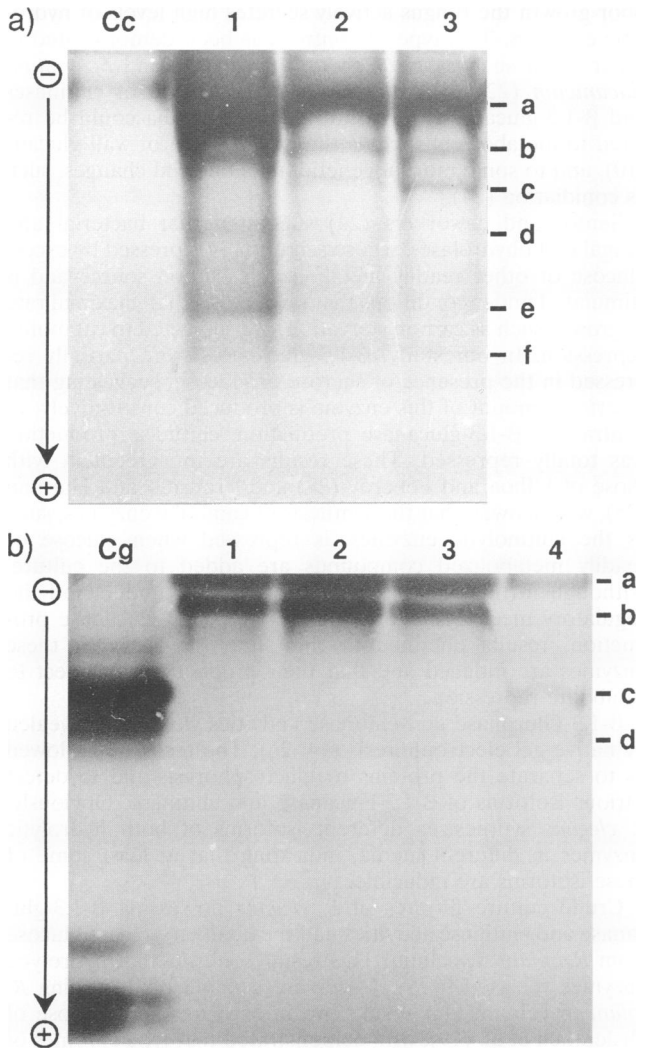

FIG. 5. PAGE of (a) chitinase and (b) β-1,3-glucanase activities secreted by *S. elegans* grown on media containing either chitin (lane 1), *R. solani* cell wall fragments (lane 2), no carbon source (MSM) (lane 3), or laminarin (lane 4). Samples (30 μl) of culture filtrates were loaded on gel impregnated with glycol chitin or β-1,3-glucan. Bands with chitinase and β-1,3-glucanase activities appeared as dark zones under UV illumination after staining with Calcofluor White M2R and aniline blue, respectively. Tobacco and barley intercellular fluids were used as positive controls for chitinase (Cc) and β-1,3-glucanase (Cg) activities, respectively.

secretion was significantly higher in media adjusted to pH 4 (3.3 CHU) and 5 (3.8 CHU). Although β-1,3-glucanase production was slightly higher at a pH range of 4 to 5, it was not significantly influenced by pH (Fig. 4b).

Six (a to f) and four (a to d) bands corresponding to chitinase and β-1,3-glucanase activities, respectively, were revealed on PAGE (Fig. 5). Depending on the carbon source used, different isoforms of both enzymes were detected. Chitinase isoform c was only found in culture filtrates of MSM and media containing cell wall fragments, whereas isoform d was only detected in chitin-containing medium (Fig. 5a). In the case of β-1,3-glucanases, identical electrophoretic profiles (isoforms a and b) were detected in culture filtrates of MSM and media containing chitin and *R. solani* cell wall fragments. A

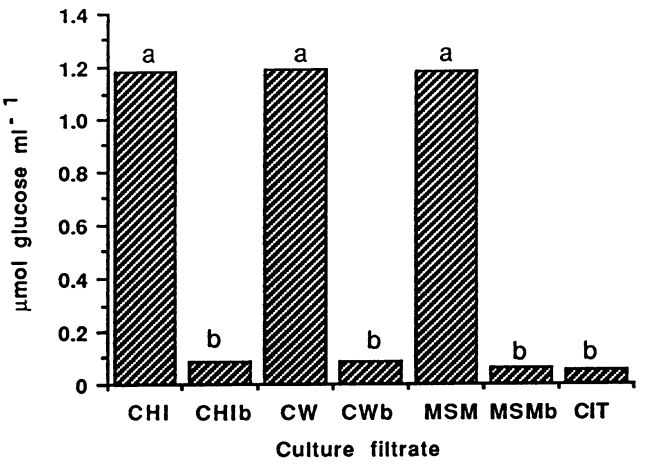

FIG. 6. Effect of *S. elegans* culture filtrates on release of glucose from *R. solani* freeze-dried mycelium. Each value represents the mean of three separate determinations. Values followed by the same letters are not significantly different (Duncan; $P \leq 0.05$). Filtrates were as follows: chitin medium (CHI); boiled chitin medium (CHIb); cell wall-containing medium (CW); boiled cell wall-containing medium (CWb); MSM; boiled MSM (MSMb); and citrate buffer (CIT).

completely different profile (isoforms a, c, and d) was detected in culture filtrate of medium containing laminarin (Fig. 5b).

Incubation of *R. solani* freeze-dried mycelium with *S. elegans* culture filtrates possessing β-1,3-glucanase activities (0.6 μmol of glucose ml of culture filtrate^{-1} h^{-1}) resulted in an important release of glucose (Fig. 6). Glucose released from mycelium incubated with culture filtrates was significantly higher than that released from mycelium incubated with either citrate buffer or boiled culture filtrates (controls). The amounts of released glucose from *R. solani* mycelium incubated with filtrates of *S. elegans* grown on MSM or on medium containing chitin or *R. solani* cell wall fragments were not significantly different.

Growth of *S. elegans* was determined on different carbon sources after 7 days of incubation (Fig. 7). Maximum growth was obtained on *N*-acetylglucosamine and sucrose-amended media compared with that obtained on other carbon sources. Growth on media containing either *R. solani* cell wall fragments or chitin was not determined because of the inability to separate the insoluble carbon polymers from *S. elegans* mycelia.

DISCUSSION

The results of this study show that *S. elegans* produces enzymes capable of degrading chitin and β-1,3-glucan, two major cell wall compounds of *R. solani*. In the presence of chitin, laminarin, *R. solani* cell wall fragments, or medium containing no carbon (MSM), *S. elegans* produced significant amounts of both chitinases and β-1,3-glucanases. These lytic enzymes, which are key enzymes in the lysis of cell walls of higher fungi, are produced by other organisms that are known to attack and parasitize fungi (11, 12, 18, 19).

Chitinase and β-1,3-glucanase production was favored by acidic pH and the presence of sodium nitrate as the sole nitrogen source. These results suggest that production of both chitinases and β-1,3-glucanases may be coordinately regulated since both enzymes were influenced similarly by similar alterations of growth parameters in the culture media. Acidic pH was also reported to be an important growth parameter in the

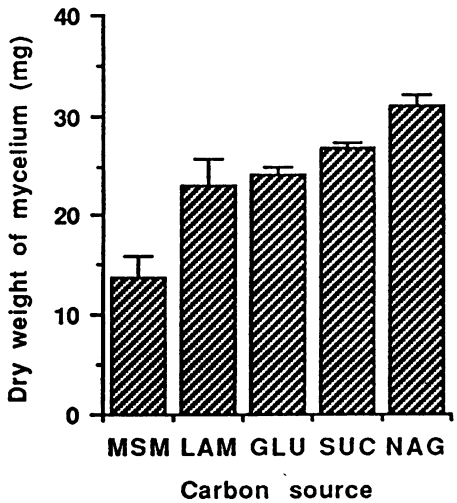

FIG. 7. Growth of *S. elegans* on different carbon sources. Mycelia grown on different carbon sources were collected after 7 days of incubation and dried at 70°C for 4 days. Growth was estimated as mycelial mass (in milligrams). Each value represents the mean of three separate determinations. Carbon sources (1 mg ml^{-1}) were as follows: minimal synthetic medium with no carbon source (MSM); laminarin (LAM); glucose (GLU); sucrose (SUC); and *N*-acetylglucosamine (NAG).

production of chitinases and β-1,3-glucanases in the mycoparasite *Trichoderma harzianum* (11) and in thermophilic *Streptomyces* species (16), respectively. Why *S. elegans* favored higher enzyme production on sodium nitrate than on other nitrogen sources is not clear, although no correlation was found between the amount of nitrogen calculated in each source (expressed in millimolarity) and enzyme production. Similarly, Butt and Ghaffar (6) showed that among seven different nitrogen sources *Stachybotrys atra* produced maximum amounts of toxins when it was grown on sodium nitrate as the sole nitrogen source.

Media containing chitin or laminarin as carbon source supported high production of chitinases and β-1,3-glucanases, respectively. Similarly, Hadar et al. (15) and Elad et al. (11) showed that the production of these enzymes in *T. harzianum* is favored by chitin and laminarin. In addition, our study shows that the production of β-1,3-glucanases was much higher than that of chitinases in medium containing cell wall fragments. These results may suggest that β-1,3-glucanases could be more important than chitinases in the degradation of *R. solani* cell walls. This is not surprising since cell walls of *R. solani* are known to contain significantly more β-1,3-glucan polymers than chitin (3, 15). Also, a significant higher production of β-1,3-glucanases in medium amended with cell walls than in media containing accessible carbon polymers (e.g., laminarin) probably suggests that different cell wall compounds of *R. solani*, or a combination of cell wall carbohydrates such as chitin and β-1,3-glucan, influence the production of glucanases. Similar results were reported by Ordentlich et al. (19), who showed that β-1,3-glucanase of the parasite *Serratia marcescens* was produced only when the bacterium was grown on a mixture of chitin and laminarin.

We also found high levels of chitinases and, particularly, β-1,3-glucanases when the mycoparasite was deprived of a carbon source (i.e., on MSM) and its growth was low (Fig. 7). Although the exact reasons for this remain to be established, it seems probable that in conditions of carbon starvation and poor growth the fungus actively secretes high levels of hydrolytic enzymes. This type of control has been demonstrated in other fungi such as *Neurospora crassa* (10), and *Sclerotium glucanicum* (22). Alternatively, the production of chitinases and β-1,3-glucanases in carbon-deprived media could be related to metabolism, such as the mobilization of wall glucans (10), and to some morphogenetic functions and changes, such as conidiation (24).

Santos and coworkers (24) suggested that bacterial and fungal carbohydrolases, whose secretion is repressed by excess glucose or other readily metabolizable carbon source and is stimulated under conditions that favor a less than maximal rate of growth such as carbon starvation, are subjected to catabolite repression. In our study, β-1,3-glucanases were partially repressed in the presence of sucrose or glucose, suggesting that a certain amount of this enzyme is produced constitutively. In contrast to β-1,3-glucanase production, chitinase production was totally repressed. These results are in agreement with those of Ulhoa and Peberdy (28) and Tronsmo and Harman (25), who showed that the synthesis of catabolic enzymes, such as the chitinolytic enzymes, is repressed when glucose or readily metabolized compounds are added to the culture. Although further analyses must be made to understand the regulatory mechanisms of chitinase and β-1,3-glucanase production, results obtained in this study suggest that these enzymes are induced and that their production is subject to catabolite repression.

β-1,3-Glucanase and chitinase activities were also revealed by native gel electrophoresis (14, 26). The technique allowed us to separate the proteins by electrophoresis and to detect various isoforms of β-1,3-glucanase and chitinase. Obviously, *S. elegans* synthesizes different isoforms of both hydrolytic enzymes in different media, indicating that at least some of these isoforms are inducible.

Crude culture filtrates of *S. elegans* possessing β-1,3-glucanase and chitinase activities had the ability to release glucose from *R. solani* mycelium. This result confirms that hydrolytic enzymes released by *S. elegans* are capable of degrading *R. solani* mycelium. Our results are in agreement with those of Ordentlich et al. (19), who demonstrated that lytic enzymes of *Serratia marcescens* degrade *Sclerotium rolfsii* mycelium and purified cell walls.

To our knowledge, this is the first report on the effect of physiological conditions on the production of extracellular chitinases and β-1,3-glucanases by *S. elegans*. The next step in this study is to purify the enzymes to homogeneity and characterize them.

ACKNOWLEDGMENTS

We thank G. Turhan, University of Ege, Ege, Turkey, for providing the strain of *S. elegans* and acknowledge the excellent assistance of S. Elouakfaoui and A. Goulet.

This work was supported by the Natural Sciences and Engineering Research Council (S. H. Jabaji-Hare) and partly by the Conseil des Recherches en Pêche et en Agro-Alimentaire du Québec (S. H. Jabaji-Hare and P. M. Charest).

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