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Characterization of Chitinase in Oak Tissues and Changes in Its Activity Related to Water Stress and Inoculation with *Hypoxylon atropunctatum*

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Chitinase activities from Shumard oak tissues were determined to study changes in chitinase activities related to water stress. The enzyme extracted in sodium acetate buffer (0.1M, pH 4.5) was assayed by a colorimetric method. In addition, the fungal hyphae of *Hypoxylon atropunctatum* in xylem tissues of oak were observed through scanning electron microscopy. The enzyme in oak tissues was mainly endochitinase, and optimum pH for enzyme activity was 5. Specific chitinase activities from both of stems held under high relative humidity (ranges of 0.63-1.11 *p*Katal/μg of protein) and stems held under low relative humidity (ranges of 0.41-0.99 *p*Katal/μg of protein) were significantly increased following fungal inoculation with *H. atropunctatum*. However, there was no significant difference in chitinase activities between tissues held under high and low humidities, which might be due to fungal chitinase. Scanning electron microscopy showed holes in fungal hyphae in the xylem tissues of stems held under high humidity but not in the stems held under low humidity, suggesting that hyphae might be hydrolyzed by plant hydolases such as chitinase.

Keywords **:** chitinase, *Hypoxylon atropunctatum*, oak tissues, stress-related decline, water stress.

The fungus *Hypoxylon atropunctatum* (Schw. ex Fr.) Cke. possibly has a role in the stress-related decline and death of oak species in the southern United States (Tainter et al., 1983; Thompson, 1963). The fungus attacks the sapwood and the phloem and eventually causes decay. Bassett and Fenn (1984) isolated *H. atropunctatum* from the living tissues of healthy-appearing oaks. They reported that the fungus was obtained from 57% of the branches and 11% of the trunks of healthy black and white oaks sampled. Also, after girdling, 77% of 108 black oaks and 70% of 88 white oaks developed stromata of *H. atropunctatum* within 5 months. From these data, they suggested that increased incidence of this fungus after host stress might be explained by rapid development of *H. atropunctatum* from latent infections.

Recent research revealed that the fungus showed little or no growth in oak stem tissues at 83% relative water content (water potential of about −0.9 MPa) but grew rapidly when water content dropped to about 71% relative water content (about −1.7 MPa) (Mason, 1990).

Both endo- and exochitinases have been found in plants (Nichols, 1980; Powning and Irzykiewicz, 1965). Endochitinase cleaves randomly within poly-*N*-acetylglucosamine to release *N*-acetlyglucosamine oligomers and exochitinase cleaves these oligomers to release *N*-acetylglucosamine (Boller et al., 1983). Many of the early reports relied on assays that measured only exochitinase activity and therefore underestimated the total chitinase activity in plants (Abeles et al., 1970; Pegg and Vessey, 1973). However, in more recent work, endochitinases have been assayed from wheat germ (Molano et al., 1979), bean (Boller et al., 1983), tomato (Pegg and Young, 1982), thorn-apple and tobacco (Broekaert et al., 1988).

There have been many reports that chitinases and β-1,3-glucanases are inducible by abiotic elicitors such as chemical compounds (ethylene and silver nitrate) (Grenier and Asselin, 1990), and biotic elicitors such as fungal cell wall fragments or by fungal attack (Boller et al., 1983; Mason, 1990). Evidence of *in vivo* lysis of pathogenic fungal hyphae in the xylem of resistant host was reported in tomato and correlated with increased activity of chitinase (Pegg and Vessey, 1973). Wargo (26) partially purified chitinase and β-1,3-glucanase from red oak (*Quercus rubra* L.), black oak (*Q. velutina* Lam.) and white oak (*Q. alba* L.) stems, and suggested that these enzymes lyse hyphal apices in healthy trees; inhibiting growth of *Armillaria mellea* (Vahl ex Fr.) Kummer as a major resistance mechanism. However, there has been no research on whether changes in chitinase activity are associated with plant water stress or are related to latent infections of plants by fungal pathogens. We hypothesized that *H. atropunctatum* might not colonize healthy oak tissues from latent infections or after inoculation in healthy unstressed stems because host hydrolases such as chitinase prevent fungus growth.

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The objectives of this research were: A) to develop an assay and characterize chitinases from Shumard oak (*Quercus shumardii* Buckl.), B) to show if changes in chitinase specific activities are related to water stress, C) to determine whether chitinase specific activities are affected by inoculation with *H. atropunctatum* in water-stressed and unstressed oak tissues, D) to compare the hyphae in xylem vessels of water-stressed and unstressed tissues inoculated with *H. atropunctatum.*

Materials and Methods

Fungus culture. A polyascospore isolate of *H. atropunctatum* isolated from stroma on the bark of a black oak (*Q. velutina* Lam.) (Bassett and Fenn, 1984) was used in this study. The fungus was stored on yeast-extract glucose agar (YEGA) slants at 4°C. YEGA contains 1.5 g yeast extract (Difco), 10 g D-glucose and 11.5 g agar per liter of distilled water. YEGA amended with 50 mg/L of streptomycin sulfate was used to culture the fungus for inoculation and isolation studies. *H. atropunctatum* was easily identified on this medium by its cultural characteristics (Bassett and Fenn, 1984).

Preparation, inoculation and incubation of stem segments. Shumard oaks used in experiments that compared the chitinase activities between water-stressed and unstressed stems were greenhouse-grown seedlings in their second growth season. The stems were cut into 18-cm-long segments and disinfested in 1% sodium hypochlorite (The Clorox Company, Oakland, CA, U.S.A.) containing 0.02% Tween 20 for 20 min with periodic agitation (Mason, 1990). The cut ends of washed stems with distilled water were sealed with liquified paraffin wax. Stems were wounded (approximately 3 mm diameter and 2 mm deep) near one end and inoculated with mycelium of *H. atropunctatum* from a 2-day-old culture. The inoculation sites were wrapped in two layers of sterile cheesecloth moistened with about 0.2 ml of sterile distilled water and sealed with two layers of Parafilm (Dixie/Marathon Co., CT, USA). The uninoculated wound sites were sealed only with two layers of Parafilm.

Stem segments inoculated or without inoculation were placed in chambers made from Com-Pack Flats (F-1020; 26×52 cm, T. O. Plastics, Inc., MN, USA) and clear vinyl domes as described by Mason (1990). Each chamber consisted of a plastic flat inside two domes. One dome was inverted and served as a reservoir for water, the other served as a lid (Mason 1990). Stems were placed on slant racks inside each chamber. The inside of the upper dome was misted twice a day with distilled water to maintain approximately 100% RH. The low humidity chamber contained no free water. Both chambers were placed under a cool-white fluorescent light bank which supplied 35-55 micro E/m^2/sec with a 16 hr photoperiod at temperatures of 22-24°C.

Measurements of relative water content. Relative water content was measured to determine the water status of the stems. Two or three pieces approximately 3 mm×2 mm deep which contained both xylem and phloem were cut from two different areas of each stem. These were weighed and then placed in a 5 cm petri dish containing 10 ml of distilled water and soaked at room temperature (19-20°C) for 24 hr. After the excess surface water was removed by blotting, the saturated weight was taken and the pieces were dried at 60°C for 24 hr then the dry weight of tissues was determined. The samples from each stem were averaged to give the relative water content of the stem.

Extraction of enzyme. Lyophilized leaves (4.6 g) that had been treated with silver nitrate (Grenier and Asselin, 1990) for 48 hr were ground in sodium acetate buffer (pH 4.5, 0.1 M) with 2 g of polyvinylpolypyrrolidone (PVP) and acid-washed sand. Stem tissues (10 g) treated with silver nitrate were sliced into small pieces and ground as above with 1.35 g of PVP and sand. The homogenate was centrifuged at 31,550 g for 30 min. The supernatant was dialyzed against distilled water for 24 hr, frozen at −20°C and lyophilized. The yields were 103 mg and 147 mg of crude enzyme preparation from leave and stems, respectively. The relationship of enzyme concentration to product formation, and pH optimum tests for the leaf enzyme were done with these lyophilized preparations. This extraction procedure was later modified by use of acetate buffer at the pH optimum of about 5.5, and the use of two centrifugation steps which gave cleaner extracts than did a single centrifugation.

Extraction of enzyme to study changes in chitinase activity related to water stress. To study whether changes in chitinase activity occur in response to water stress in unwounded stems, stem pieces (0.4 g fresh weight) including phloem and xylem were randomly cut from each water-stressed and unstressed stem. Tissues were ground in sodium acetate buffer (pH 5.5, 0.1 M) with 22.2 mg of PVP and acid-washed sand. The homogenate was centrifuged at 17,300 g for 10 min and the supernatant was centrifuged a second time. This supernatant was frozen at −20 °C until crude enzyme preparations were prepared from 30 stems.

To study changes in chitinase activity from unwounded, wounded or wound-inoculated stems, stem pieces including phloem and xylem (0.2 g fresh weight) were taken from three random sites for unwounded, three wound sites or three wound-inoculated sites on a stem. Tissue samples were about 2 mm thick and collected from the wound or inoculation sites and tissues 1 cm above and below the sites. Crude enzyme preparations were prepared as described above for unwounded stems except that 7.4 mg of PVP was used per sample. Crude enzyme preparations were frozen at −20°C until crude enzymes were prepared from 36 stems.

Chitinase assays. Chitinase was assayed following the method of Boller et al. (1983) which involves incubation of enzyme with insoluble colloidal chitin, enzymatic conversion of soluble N-acetylglucosamine oligomers to N-acetylglucosamine, and colorimetric assay of N-acetylglucosamine by the method of Reissig et al. (1955). Crude crab shell chitin (Nutritional Biochemicals) was bleached with $KMNO_4$ by the method of Berger and Reynolds (1958). Bleached chitin was solubilized in concentrated HCl and precipitated with aqueous ethanol to produce a colloidal chitin preparation (Skujins et al., 1965). Conversion of soluble N-acetylglucosamine oligomers to N-acetylglucosamine was done with almond β-glucosidase (EC 3.2.1.21, Sigma Product No. G-0395, Sigma Chemical Co.) which contains an active exochiti-

nase (Pegg, 1988).

To determine chitinase activity, enzyme solution and sodium acetate buffer (0.1 M, pH 5.5) were made to total volume of 0.4 ml in a 1.5 ml polypropylene centrifuge tube and 0.14 ml (0.98 mg) of colloidal chitin was added. The tubes were incubated in a 37°C water bath for 3 hr with agitation at 15 min intervals. The tubes were then centrifuged at 14,000 g for 5 min. A 0.3 ml aliquot of the supernatant was placed in a glass tube, and 0.1 ml of almond β-glucosidase (2 mg/ml, 9.2 units/ml) in water was added and incubated at 37°C for 1 hr. The volume was adjusted to 0.5 ml with water and the *N*-acetylglucosamine content was assayed by the method of Reissig et al. (1955) and quantitated using a standard curve produced with authentic *N*-acetylglucosamine.

Test for endo- and exochitinase activities. To determine if crude enzyme extracts contained endo- or exochitinase activities or both, N-acetylglucosamine formation was compared in assays done with and without almond β-glucosidase to convert *N*-acetylglucosamine oligomers to *N*-acetylglucosamine. These results were compared to assays containing heat-treated enzyme (100°C for 15 min). Incomplete reaction mixtures in which the various components were omitted from the assay were done to estimate their effects on A585 nm.

pH optimum. The effect of pH on chitinase activity was determined over the range 3-9. Sodium acetate buffer (0.05 M) was used for the pH range of 3 to 5, potassium phosphate buffer (0.05 M) for the pH range 6 to 7 and Tris-HCl buffer (0.05 M) for the pH range 8 to 9. Reaction mixtures were as described previously except that the almond β-glucosidase was dissolved in sodium acetate buffer (0.3 M, pH 5.0) to maintain the final pH near 5 which is optimum for activity of the exochitinase. Lyophilized crude enzyme from leaves was dissolved in 3 ml of distilled water and 0.1 ml (18.9 μg of protein) was used in each assay.

Time course. Assay mixtures (13.9 μg of protein/0.4 ml) in acetate buffer (pH 5.5, 0.1 M) were incubated from 0 to 5 hr. Chitin hydrolysis was determined at 10 min-intervals up to 1 hr and at 1 hr-intervals from 1 to 5 hr. Two replicate assays were done at each time.

Relationship of enzyme concentration to product formation. Crude enzyme from stems (221.5 g of protein/ml of sodium acetate buffer of pH 5.5, 0.1 M) was used. Reaction mixtures contained a dilution series (0-320 μl of enzyme) in sodium acetate buffer (pH 5.5, 0.1 M). Enzyme without chitin was used as an assay blank. Enzyme units were expressed as *p*Katal, which is the amount of enzyme that liberates one picomole of N-acetylglucosamine per second. Three replications were assayed for each enzyme dilution. A similar dilution series of crude enzyme (189.2 μg of protein/ml of sodium acetate buffer of pH 5.5, 0.1 M) from leaves was assayed to determine the relationship of enzyme concentration to product formation for the leaf enzyme.

Protein assay. Protein concentration was determined following the method of Bradford (1976) using protein reagent from BioRad Laboratories. A standard curve for the microassay procedure (1-20 μg) was made with bovine serum albumin. Duplicate 0.1 ml aliquots of enzyme preparation were assayed for each determination.

Samples for electron microscopy. Stems of greenhouse-grown Shumard oaks (18 cm length, 4 mm in diameter) were used. Twelve stems of oaks were placed under high humidity (about 100% relative humidity) or low humidity (about 50% relative humidity) for 3, 4 and 5 days after inoculation (Mason, 1990). As controls, six uninoculated stems were placed under high and low humidity.

After 3, 4 and 5 days, relative water content of the stems was measured and the xylem of each stem was longitudinally split and sectioned into pieces 3 mm long and 1 mm thick. Alternate pieces were either fixed for electron microscopy or plated on YEGA to recover the fungus. For stems held under high humidity for 3 days, pieces were collected up to 2.7 cm from the inoculation sites, whereas for stems held under low humidity for 3 days pieces were collected from 3 cm to 9.3 cm from the inoculation sites. Samples were taken up to 5.4 cm and from 4 cm to 10.3 cm from inoculation site for the stems held at high or low humidity, respectively, after 4 days. After 5 days incubation, samples were taken up to 5.4 cm and from 6 to 14.1 cm from inoculation site in the high and low humidity chambers, respectively. Only samples adjacent to those from which the fungus was recovered on YEGA were used for electron microscopy. The samples from uninoculated stems were taken from the middle of the stems regardless of humidity treatment.

Preparation of specimens for scanning electron microscopy. As soon as the stems were sectioned, xylem pieces were placed in a modified Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer at pH 7.4) for 2 hr under a weak vacuum. The fixed samples selected for scanning electron microscopy were washed in two changes of 0.05 M cacodylate buffer 20 min each time and post fixed for 2 hr in 1% OsO_4 in the same buffer. The tissues were washed briefly with distilled water for 1-2 min, prestained in 0.5% aqueous uranyl acetate overnight and dehydrated in a series of ethanol solutions (30%, 50%, 70%, 80%, 95%). After the 95% ethanol solution, pieces were processed with critical point drying and were sputter coated with gold. Electron micrographs were taken with an ISA 60 scanning electron microscope.

Results

Test of exochitinase and endochitinase. The data in Table 1 shows that the chitinase from stem tissues of oaks was an endochitinase. Because there was no difference in absorbance between the treatment containing enzyme and chitin and that containing heat-treated enzyme, chitin and β-glucosidase, there was no evidence of exochitinase activity in the enzyme preparation. The enzyme solution, chitin, β-glucosidase or buffer contributed little to color development.

Optimum pH of endochitinase from leaves. A pH of about 5 was optimum for activity of the endochitinase from oak leaves (Fig. 1). The enzyme was active over a broad pH range between pH 4 and 7 and maintained activity at least 3 days storage in a refrigerator.

Time course of endochitinase activity. Endochitinase

activity in a crude enzyme extract from stem tissues was not linear over time (Fig. 2). From 0 to 1 hr, hydrolysis occurred rapidly, but gradually slowed.

Effect of enzyme concentration on product formation. Endochitinase activity was not linearly related to the increase of the concentrations of crude enzyme extract from stems (Fig. 3). Similar results were found with the enzyme extract from leaves. From the plot of enzyme concentration versus product formation, the initial slope (tangent to the curve) was visually estimated. This slope was used to estimate enzyme units (*p*Katal) at infinite dilution for each enzyme preparation (Boller et al., 1983).

Changes in chitinase activities in relation to water stress in unwounded stems. The activities of chitinase in stem tissues held under high humidity or low humidity for up to 5 days were compared to see whether changes in chitinase activities were related to water stress. The relative water content of stems under low humidity were significantly lower than that of stems held under high humidity throughout the period (Fig. 4). The specific activities of chitinase from oak stem tissues held under either high or low humidity were, however, not significantly different either within a day or between days (Fig. 5). Ranges of specific activity were 0.10-0.57 *p*Katal/μg of protein (high humidity) and 0.11-0.33 *p*Katal/μg of protein (low humidity).

Chitinase activities in unwounded, wounded and inoculated stems in relation to water stress. Chitinase activities in oak tissues incubated under high humidity or low humidity for 3.5 days were compared to see whether changes in chitinase activity were caused by wounds and/or inoculation. There were no differences in specific activities of chitinases between unwounded and wounded stems held under either humidity regime (within a humidity treatment), but specific activities were significantly higher in inoculated stems than either unwounded or wounded stems under both humidity treatments (within a humidity treatment) (Fig. 6). There were no significant differences in the specific activities of chitinase across the humidity treatments for unwounded, wounded or inoculated stems (Fig. 6). The differences in relative water content between stem segments held under high or low humidity were significantly different (*t* test, $P = 0.05$) (Fig. 7).

Table 1. Test of endochitinase and exochitinase activities in extracts from stems of Shumard oak and contributions of enzyme, chitin, β-glucosidase, buffer and heat-treated enzyme to the color development.

Treatments[a]	Absorbances at 585 nm[b]
Enzyme+chitin + β-glucosidase	0.120
Enzyme + chitin	0.024
Heat-treated enzyme+chitin + β-glucosidase[c]	0.024
Heat-treated enzyme + β-glucosidase	0.020
Enzyme + β-glucosidase	0.015
Chitin + β-glucosidase	0.007
Enzyme	0.009
Chitin	0.006
β-glucosidase	0.005
Buffer	0.005

[a] Assay mixtures (0.54 ml total volume) were incubated at 37°C for 3 hr. The buffer was 0.1M sodium acetate pH 5.5. Water, buffer or both were used as appropriate to maintain the assay volume when components were left out.

[b] A585 nm was set at 0.000 with disitilled water. Three replications were done (The range of variation was 0-25%).

[c] Enzyme solution was placed at 100 °C for 15 min.

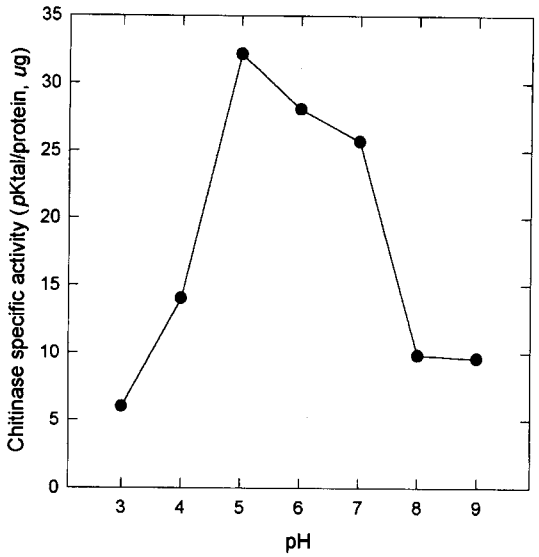

Fig. 1. Effect of pH on the activity of chitinase from leaf tissues of Shumard oak. Data points represent the means of three replicates for each pH.

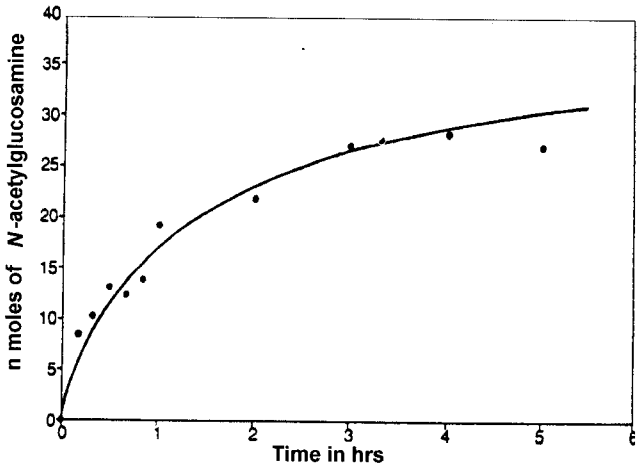

Fig. 2. Effect of incubation time at 37°C on product formation by chitinase from oak stem tissues. Assay mixtures contained 13.9 μg of total protein. Data points represent the means of two replicates for each time.

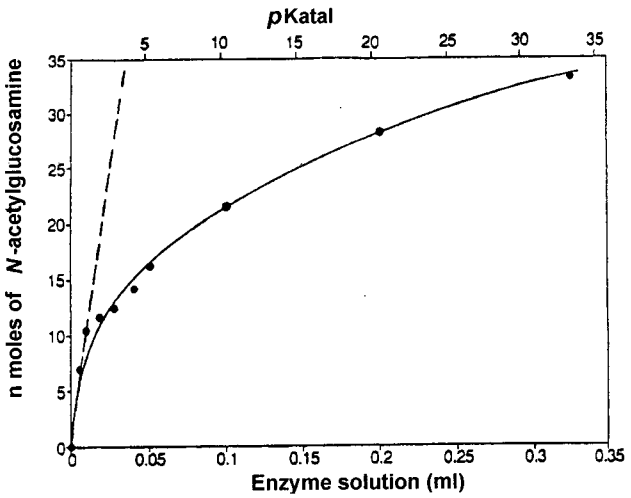

Fig. 3. Effect of enzyme concentration on activity of chitinase. Reaction mixtures were incubated for 3 hrs at 37°C. Data points represent the means of three replicates for each dilution of enzyme. Initial slope (---) was used for the calculatin of enzyme units.

Ranges of specific activities (*p*Katal/μg of protein) for stems under high humidity were 0.26-0.53 for unwounded, 0.31-0.56 for wounded, and 0.41-0.99 for inoculated and those under low humidity were 0.29-0.59 for unwounded, 0.41-0.75 for wounded, and 0.63-1.11 for inoculated. Ranges of relative water content were 79.3%-90.2% (high humidity) and 43.3-72.1% (low humidity).

SEM comparison of hyphae in xylem vessels of stems held under high or low humidity. The hyphae with the average distance of 2.7 cm in xylem vessels of stem segments under high humidity (relative water content of 77.7%) were generally smaller (mean diameter: 0.41 μm) than those with the average distance of 8.9 cm grown under low humidity (relative water content of 56.3%) (mean diameter: 0.75 μm). In addition, the hyphae grown in stems at high humidity showed a somewhat withered appearance and had sunken areas as well as holes indicating signs of degradation. Hyphae grown under both high and low humidity penetrated through scalariform end plates of xylem vessels. To quantify the occurrance of hyphae with holes, 14 hyphae randomly chosen in xylem vessels from each treatment were examined. In the xylem vessels grown held under high humidity, 78% of hyphae had holes while only 22% of hyphae in vessels of stems held grown under low humidity had the holes (Fig. 8).

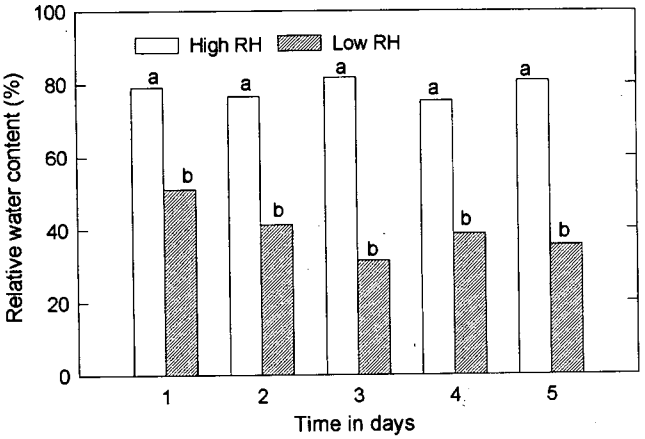

Fig. 4. Changes in relative water content of stem segments held under high humidity (about 100%) or low humidity (about 50%). For each day, bars with different letters are significantly different (*t* test, *P*=0.05). There were three replicates per treatment.

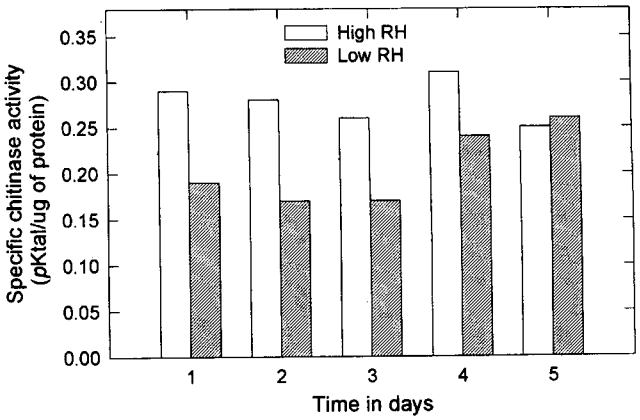

Fig. 5. Changes in specific activity of chitinase related to relative water content (see Fig. 4). There was no significant difference within (*t* test, *P*=0.05) or between days. There were six replicates per treatment.

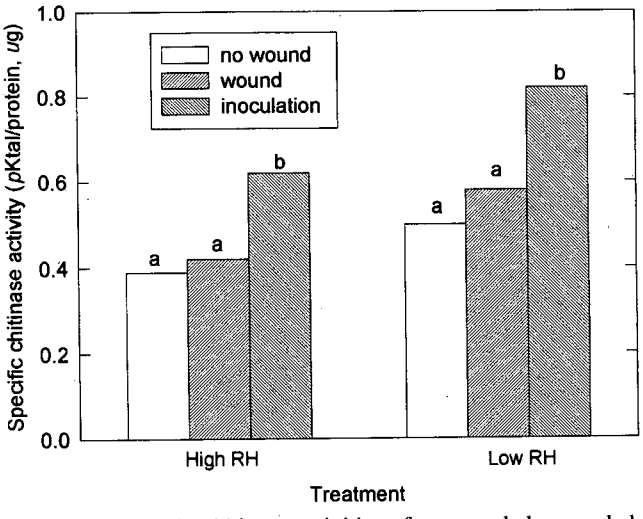

Fig. 6. Changes in chitinase activities of unwounded, wounded, wound-inoculated stem segments held under high or low humidity for 3.5 days. Within a humidity treatment, bars with the same letters were not significantly different. Between humidity treatments, bars with same letter were not significantly different (*t* test, *P*=0.05). There were six replicates per treatment.

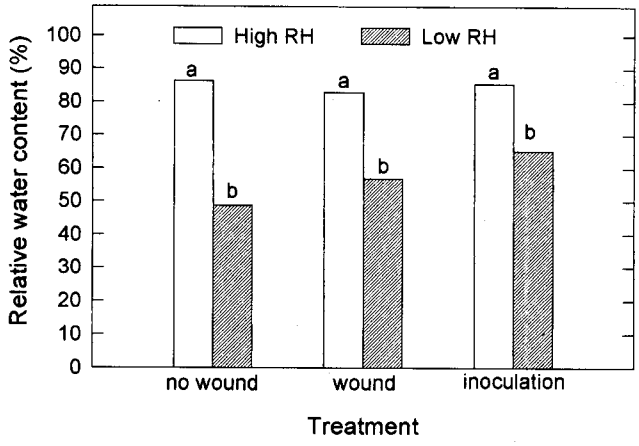

Fig. 7. Relative water content of stem segments held under high humidity (about 100%) or low humidity (about 50%) for 3.5 days. Stem segments were unwounded, wounded or inoculated with *H. atropunctatum*. For each treatment (unwounded, wounded or wound-inoculated), bars with different letters are significantly different (*t* test, P=0.05). There were three replicates per treatment.

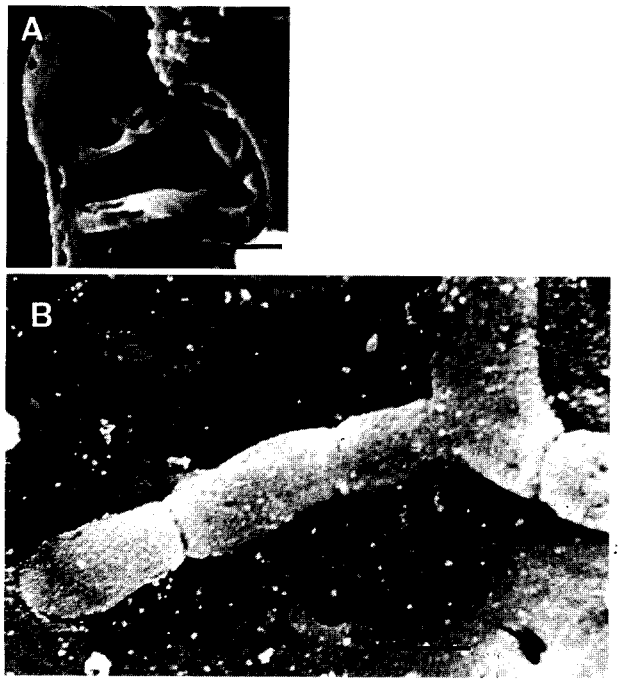

Fig. 8. Scanning electron micrographs showing the hyphae of *H. atropunctatum*. (A) Several holes suggesting partial degradation of hypae in a xylem vessel of stem segements under relative high humidity. Scale bar = 1 μm. (B) Healthy hypae without holes in a xylem vessel of stem segements under relative low humidity. Scale bar = 1 μm.

Discussion

The chitinase from oak stem tissues assayed in the present studies was an endochitinase. Exochitinase activity was not detected (Table 1). The assay by Wargo (1975) depended upon the activity of an exochitinase in his extracts, therefore, he probably underestimated the total chitinase activity in oak tissues. There have been many reports about assays specific for plant chitinases, but most of these researches dealt with exochitinase activities which did not reflect total chitinase activity (Abeles et al., 1990; Nichols et al., 1980; Pegg and Vessey, 1973; Pegg and Young, 1981). However, Powning and Irzykievicz (1965) assayed endochitinase which they described as chitinase I and II.

The optimum pH of 5 for endochitinase from Shumard oak leaf tissues (Fig. 1) was similar to that (pH 5.2) of the chitinase reported by Wargo (1975), although he did not report the optimum pH of chitinase from only oak tissues. This is lower than the pH optimum of 6.5 for the endochitinase from bean reported by Boller et al. (1983). Wargo (1975) also reported that chitinases from stem bark of red oak and sugar maple maintained high activity over the pH range 5 to 6. This is similar to the chitinase from leaf tissues of Shumard oak which showed high activity from pH 4.5 to pH 7 in this study. Chitinase maintains high activity over a broad pH range, although the pH optimum differs from species to species (Bollard, 1958; Boller et al., 1983; Wargo, 1975).

Product formation by Shumard oak endochitinase was neither linear over time nor with enzyme concentration (Fig. 2 and 3). This was similar to the results which Boller et al. (1983) reported for the endochitinase from bean and Molano et al. (1977) reported for wheat germ endochitinase. The latter workers considered this to be due to the polydisperse, heterogeneous nature of the chitin substrate; Portions of which were hydrolyzed at different rates. Their results suggested that subtrate exhaustion or enzyme instability might not be responsible. At pH 6.5, chitinase from bean was stable at temperatures up to 40°C with less than a 10% loss of activity in 6 hrs (Boller et al., 1983).

Chitinase specific activities were not increased by wounding in stem tissues held under high or low humidities but increased about 50% after inoculation and incubation under high or low humidity (Fig. 6). This implies that the fungus might act as a biotic elicitor to induce chitinase and that oak stem tissues respond to this fungal infection but not to wounding. Preliminary results showed that chitinase activity also increased after treatments with silver nitrate solution (100 μM) (data not shown). Boller et al. (1983) reported that chitinase was not induced in bean leaves by mechanical wounding *in situ*, but only in a system with a closed atmosphere where wound ethylene accumulated. The increased activities of chitinases in inoculated stems might originate from both fungal chitinases and plant chitinases.

Chitinase specific activities were not significantly differ-

ent for unwounded stems held under high or low humidities for up to 5 days, indicating that constitutive chitinase activities were stable over this time period regardless of the relative water content. Chitinase specific activities (0.24 *p*Katal/μg of protein on average) (Fig. 5) in tissues of unwounded stems collected in September, 1991 were lower than those (0.45 *p*Katal/μg of protein on average) (Fig. 6) in tissues of unwounded stems collected in May, 1992. Tissues collected in September, 1991 were from seedlings entering dormancy while those collected May, 1992 were from actively growing trees. Bier's work (1964) indicated that actively growing seedlings were more resistant to invasion by secondary canker pathogens than were dormant trees. The higher chitinase activity in the trees collected in May than in September implies that chitinase may be involved in the resistance mechanism of oak trees growing actively, as Bier (1964) suggested.

The specific activities of chitinase were not affected by water stress. Although chitinase specific activities increased following fungal infection of stems incubated under high or low humidity, the trend was the same. However, scanning electron microscopy showed more holes in hyphae inside xylem vessels of stems held under high humidity than those under low humidity. This observation suggests that plant hydrolases might be involved in the resistance of oak tissues to *H. atropunctatum*. Holes observed in *H. atropunctatum* were similar to those reported by Ordentlich *et al.* (1988) as an evidence of partial degradation of *Sclerotium rolfsii* hyphae possibly caused by chitinase from *Serratia marcescens*. They showed not only holes in hyphae of *Sclerotium rolfsii*, but also the accumulation of bacterial cells around the hyphae of *Sclerotium rolfsii*. McPartland and Schoeneweiss (1984) reported swelling, bursting or holes in the tips of hyphae in unstressed white birch stems and suggested that they might result from an active biochemical host defense response. Wargo (1975) reported that chitinase from oak caused *in vitro* hydrolysis of cell walls of *Armillaria mellea* suggesting that chitinase might be involved in a resistant mechanism of oak trees.

Assays did not reveal a relationship between the specific activity of chitinase and water content or fungal infection of stems. However, there are several aspects to be considered in designing further research on this topic. First, the location of chitinase was not studied in this research. Chitinase may be involved in inhibiting fungal growth at specific locations or in certain tissues, but this can not be determined when chitinases are extracted from plants. Therefore, simply comparing chitinase activities between unstressed and stressed stem tissues can not answer why the fungal growth was inhibited in the unstressed stem tissues. More available nutrient to be utilized by the fungus in the stems held under low humidity than high humidity might let the fungus overcome inhibition by chitinase. Mason (1990) reported that starch hydrolysis occurred to a limited extent in water-stressed stems and suggested that starch could supply a carbon and energy source. Also, electrolyte leakage from host cell and host cell metabolites released by disruption of membranes in the water-stressed tissues may be a factor that enables the fungus to grow rapidly (Mason, 1990) and to overcome inhibition by chitinase.

The present study only assayed endochitinase with an acidic pH optimum in crude enzyme preparations. Further studies are needed to purify chitinases from oak stems and determine whether they have different properties. More detailed experiments are needed to correlate increased chitinase activity and changes in fungal growth. Also, plants have not only chitinases but also β-1,3-glucanases as antifungal hydrolases that are suggested to be part of a plant's defensive mechanisms (Mauch et al., 1988; Wargo, 1975). In addition, to understand the resistant mechanism of oak stems, the location of chitinase needs to be studied in unstressed and water-stressed oak stems. The sources of increased chitinase specific activity following fungal infection with *H. atropunctatum* remain to be further studied.

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