Responses of American and Chinese Chestnut to *Cryphonectria parasitica* and Ethylene

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ABSTRACT. An understanding of the complex interactions between resistant or susceptible chestnut to *Cryphonectria parasitica* may provide a basis for early screening for genotype resistance and, perhaps assist future efforts to transform American chestnut. Tests did not confirm the presence of preformed inhibitors in Chinese chestnut bark. Studies, therefore, were undertaken to determine if compounds induced during challenge may play a definitive role in host resistance. Stem segments of American and Chinese chestnut were inoculated with conidia of *C. parasitica.* For a cell-free challenge, stem segments were incubated in the presence of ethylene. After incubation, bark was freeze-dried, ground, and extracted in hot water, polar or non-polar organic solvents, or in buffer. Hot water extracts of neither species were antifungal. Bioassay results of extracts soluble in organic solvents have been inconsistent. Immunoblots of proteins separated by SDS polyacrylamide gel electrophoresis (PAGE) showed the induction of b-1,3-glucanase and chitinase in both hosts after living or non-living challenge. Isoforms of chitinase induced in Chinese chestnut differed from those induced in American chestnut. Intraspecific isoforms of chitinase were similar for living or non-living challenges and for trees challenged in the dormant or growing seasons. These hydrolases have been shown to lyse the cell walls of other fungal plant pathogens. Native proteins from ethylene-challenged bark of both species, but not from unchallenged bark or boiled challenged bark, lysed the hyphae of C. *parasitica.* In this study, protein extracts from Chinese chestnut bark were more antifungal than those from equal amounts of American chestnut bark.

Among *Castanea* spp., American chestnut, *Castanea dentata* (Marsh.) Borkh. is considered the most susceptible to the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr, and Chinese chestnut, *C. mollissima* Bl., the most resistant (12). An understanding of the mechanism(s) of resistance would add to our basic knowledge and may provide a basis for the early screening of genotypes that are being bred for blight resistance (8) or for future efforts to transform American chestnut.

Research previously focused on preformed compounds, i.e., compounds already present at the time of inoculation, to explain the resistance of Chinese chestnut to blight. Nienstaedt (19) reported that hot water extracts of Chinese chestnut inhibited mycelial growth of *C. parasitica* to a greater extent than similar extracts from American chestnut bark. Bazzigher (1), however, reported that enzymes produced by the blight fungus can degrade bark tannins of both species. Specific bark tannins of American and Chinese chestnut, furthermore, were utilized by *C. parasitica* as sole carbon sources (10). A fraction of a petroleum ether extract from bark of Chinese but not American chestnut totally inhibited the mycelial growth of *C. parasitica.* This fraction was reported to contain a long-chain unsaturated fatty acid (22). Fungal polygalacturonase was inhibited more by phenolic and proteinaceous extracts from Chinese than from American chestnut bark (18).

Compounds induced in response to chestnut blight have received relatively little attention as compared to studies on the effects of preformed compounds. Histological studies showed that lignification occurred in both hosts prior to wound-periderm formation (13). Suberization of lignified cells was reported as part of the response of American chestnut to wounding (3). Ethylene was induced in both hosts in response to wounding or infection (14). This gaseous hormone has been implicated in the regulation of plant defense (9).

Reports on the induction of antifungal compounds in response to challenge, e.g., phytoalexins, antifungal hydrolases (17), are lacking for the *Castanea—Cryphonectria* pathosystem. The objective of this study was to determine if such antifungal compounds are induced and if they can be related to the observed resistance of Chinese chestnut to blight.

MATERIALS AND METHODS

Challenge of host tissue. Stems of American and Chinese chestnut (ca. 5 cm diameter) were collected from Powell and Woodford Counties, Ky., respectively, during the dormant and growing seasons. Stem segments, 12 cm in length, were inoculated by immersing them in conidial suspensions of *C. parasitica* strain EP 155 (ATCC No. 38755) or its isogenic hypovirulent strains, EP 905 or EP 915, which contain hypovirulence factors from Tennessee or Virginia designated as HT2 and HV1 , respectively (11). Conidia were harvested from colonies grown on potato dextrose agar (Difco, Detroit, Mich.), amended with 100 mg/1 methionine and 1 mg/1 biotin, and ranged from 4 x 10^7 — 1 x 10^8 conidia/ml. Stem segments were incubated at room temperature in sterilized vacuum desiccators that were ventilated continuously (ca. 50 ml/min) with humidified air to prevent buildup of metabolic gases and to reduce desiccation.

For a cell-free challenge, stem segments were incubated in vacuum desiccators that were ventilated as above

with humidified air containing ethylene (ca. 10 ppm). Bark frozen at the time of collection, as well as stem segments incubated in humidified air alone, served as controls for both fungal and cell-free challenges. Incubation times varied from 6-14 days for fungal challenge and 3-6 days for ethylene challenge.

Processing of bark. After incubation, the periderm was removed and the remaining bark was stripped from stem segments and freeze-dried. Bark samples then were ground with dry ice in a Wiley mill to pass a 20-mesh screen. All ground bark samples were stored in septum-sealed, evacuated glass bottles at —20 C.

Extraction of bark with water and organic solvents. Some ground bark samples were extracted in hot water as described by Nienstaedt (19). Other ground samples of challenged and unchallenged bark were extracted successively in three volumes of methanol and then hexane. The solvent used for each extraction was approximately ten times the weight of the ground bark. A rotary shaker at ca. 200 rpm was used to facilitate extraction. Extracts with each solvent were pooled and concentrated for subsequent bioassay.

Extraction and electrophoresis of bark protein. Freeze-dried, ground-bark meal (0.2 g) was ground with a cooled mortar and pestle containing polyvinylpyrrolidone (pvpp), at one-half the weight of the bark sample, with 0.0625 M Tris-HC1 buffer, pH 6.8, containing 1% sodium dodecyl sulfate (SDS), 1% mercaptoethanol, and 1.5 M urea. This suspension was centrifuged for five minutes at 16,000 g. The concentration of SDS-extracted proteins in the supernatant was determined by the Lowry (16) assay. The supernatant, ca. 60 fig protein, was separated by SDS polyacrylamide gel electrophoresis (PAGE) with molecular weight standards (Bio-Rad, Richmond, Calif.) on a 4% stacking gel and a linear gradient 12.5-20% polyacrylamide resolving gel. Electrophoresis was conducted in a Hoefer SE series 600 vertical slab gel unit (HSI, San Francisco, Calif.) using the discontinuous buffer system of Laemmli (15) for ca. 3 h at 30 mA. Some gels were stained with Coomassie brilliant blue R-250 for viewing. Proteins from other SDS PAGE gels were transferred to nitrocellulose membranes using a trans-blot electrophoretic transfer cell (Bio-Rad) overnight at 30 V. After blocking nonspecific binding sites for immunoglobulins with nonfat milk, membranes were incubated with antibodies to chitinase or b-1,3-glucanase . Immunoblotting was completed by incubating nitrocellulase membranes with anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, St. Louis, Mo.) and then a reaction mixture containing MgC12, nitro-blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (4).

Native protein was extracted from ground bark samples in the cold in 0.1 M potassium phosphate buffer, pH 7.0, containing pvpp, and 0.1 M sodium ascorbate. Three extractions of ca. 0.5 g bark in 5 ml of buffer were pooled and centrifuged at 12,000 g. Protein in supernatants was precipitated with ammonium sulfate (95% saturation at 0 C). Precipitated proteins were redissolved in 0.1 M potassium phosphate buffer, pH 7.4 and dialyzed (12-14 x 10^3 MWTC) overnight in the same buffer at 4 C. Concentration of native proteins was determined by the Bradford (6) assay. Electrophoresis of native proteins was conducted in the absence of SDS in 10% polyacrylamide gels as described above. Chitinase was detected by incubating native-protein gels with an overlay gel containing glycol chitin. The overlay gel was removed from the resolving gel, stained with calcofluor white, and viewed under UV light for bands of chitinase activity. The resolving gel was then incubated with laminarin and stained with 2,3,5-triphenyltetrazolium chloride for the detection of b-1,3-glucanase (20).

Bioassay of extracts. Bioassays were conducted to determine if bark extracts contained components antagonistic to mycelial growth or conidial germination of C. *parasisica.* Mycelial growth rate was monitored after addition of hot-water extracts to PDA or water agar as described by Nienstaedt (19). Protein extracts were assayed by placing them into wells (6 mm diameter) cut from uncolonized PDAmb in advance of growing mycelium. Other extracts were tested for inhibition of conidial germination by adding them to filter paper disks (7 mm diameter) mounted on pins and, after evaporation of solvent, placing disks on the surface of minimal-medium agar (21) containing conidia (10^6-10^7 /ml) that were added when the autoclaved medium cooled to ca. 50 C.

RESULTS

Bioassays of hot water extracts from unchallenged bark of both species did not show a reduction of mycelial growth on Chinese chestnut extracts as compared to similar extracts from American chestnut or controls grown on culture media. Furthermore, bioassays of methanol or hexane extracts of unchallenged bark showed no greater antifungal activity in Chinese chestnut extracts. Inconsistent results were obtained with some extracts from challenged bark, but these await further study to determine if they can be related to the resistance of Chinese chestnut.

Two additional bands, sized at ca. 30 and 33 kDa, were observed on Coomassie-stained SDS PAGE gels from challenged bark of both species (Figure 1). These are the sizes reported previously for chitinase and b-1,3-glucanase , produced by other plant species (5). Immunoblots of these new bands, with antibodies for both enzymes, were positive for challenged bark of both Chinese and American chestnut (Figures 2A, 2B). Isoforms of chitinase could be demonstrated on PAGE separated native protein gels that were overlayed with a gel containing glycol chitin (Figure 3). Isoforms of chitinase induced in Chinese chestnut differed from those induced in American chestnut. Intraspecific isoforms of chitinase were similar for living and cell-free challenges and for trees challenged in the dormant or growing season.

Bioassays of native proteins from ethylene-challenged bark of both species but not from unchallenged bark or boiled challenged bark, showed inhibition of mycelial growth. Protein extracts from Chinese chestnut, however

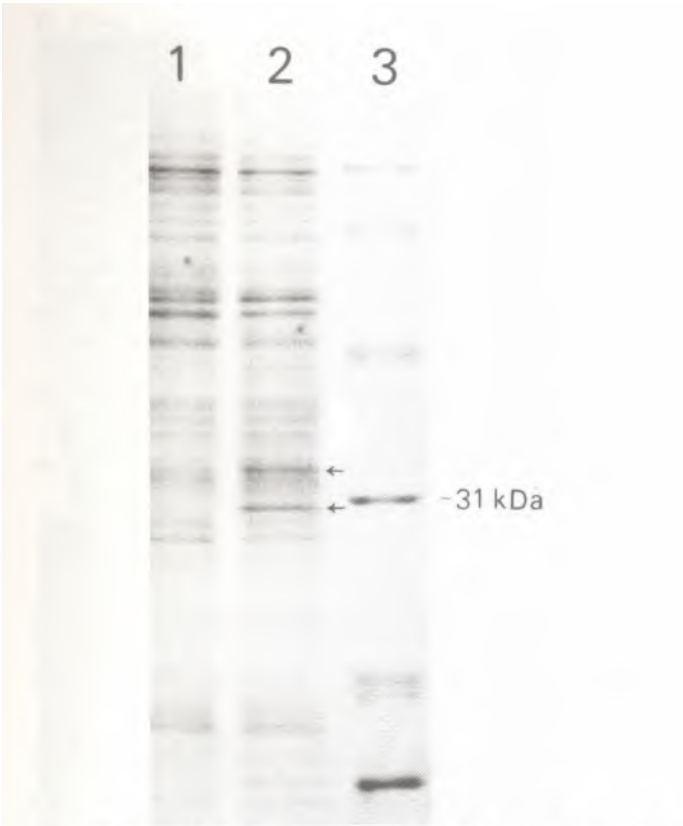

Figure 1. SDS-PAGE of proteins extracted from Chinese chestnut bark according to Laemmli (15) using a 4% stacking and 12.5 – 20% linear gradient resolving gel at 30 mA for ca. 3 h. Lane 1, extracts of bark incubated for 6 days in air; Lane 2, extracts of bark incubated for 6 days in air containing ca. 10 ppm ethylene. The appearance of two new proteins at ca. 30 and 33 kDa is indicated by lower and upper arrows, respectively; Lane 3, molecular weight standards (kDa top to bottom) 97.4, 66.2, 42.7, 31, 21.5, and 14.4.

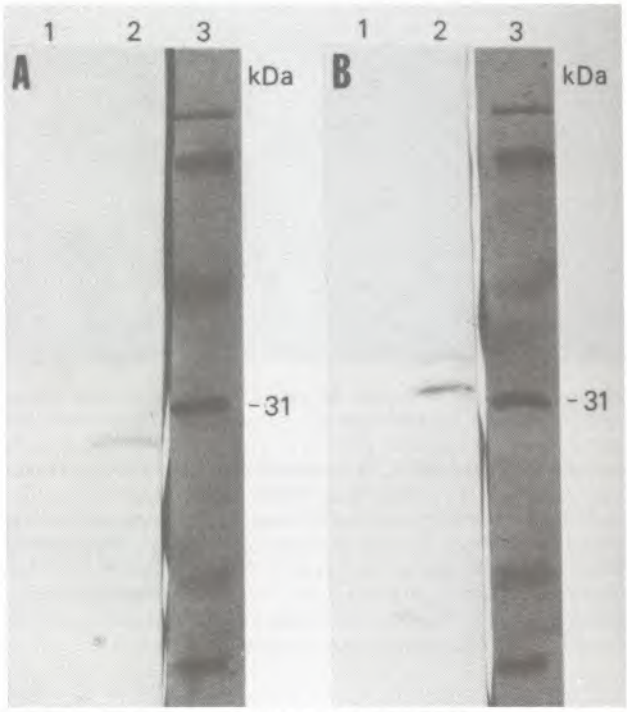

Figure 2. Western blots of SDS-PAGE separated proteins extracted from Chinese chestnut bark and developed according to Blake et al. (1984) with antibodies to A. chitinase and B. β-1,3-glucanase. Lanes 1, extracts of bark incubated for 5 days in air; lanes 2, extracts of bark incubated for 5 days in air containing ca. 10 ppm ethylene; Lanes 3 molecular weight standards (kDa top to bottom) 97.4, 66.2, 42.7, 31, 21.5 and 14.4.

were more antifungal than those from equal amounts of American chestnut bark (Figure 4). Hyphae of *C. parasitica*, were lysed by native protein extracts from ethylene-challenged bark (Figures 5A, 5B).

DISCUSSION

The induction of phytoalexins or antifungal hydrolases as possible mechanisms of resistance of Chinese chestnut to blight has not been reported previously. Although we have gained little evidence to support the occurrence of phytoalexins, this area is worth additional investigation.

We were successful in demonstrating the induction of chitinase and b-1,3-glucanase in both chestnut species host material collected during the dormant and growing seasons. This induction appears to be a common host response to injury, infection, and ethylene (5). Chitinase and b-1,3-glucanase have been implicated in the lysis of chitin and b-1,3-glucan, two major fungal cell wall components (17). While the isoforms of chitinase seem to differ between American and Chinese chestnut, we have not yet been successful in separating isoforms of ,3-1,3-$_g$lucanase. Isoform differences may be potentially useful genetic markers and also indicate difference in biological activity.

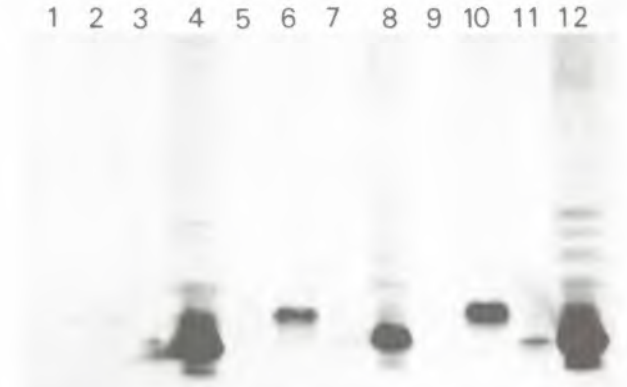

Figure 3. Chitinase activities in extracts of American and Chinese chestnut bark after PAGE of native proteins and incubation with an overlay gel containing glycol chitin according to Pan et al. (20). Each lane contains extracts from 40 mg of dry bark. Lanes 1, 2, 5, 6, 9, 10 are from American chestnut whereas lanes 3, 4, 7, 8, 11, 12 are from Chinese chestnut. Lanes 1–4, 5–8, and 9–12 are from trees harvested on 12/1/89, 6/22/90, and 3/9/90, respectively. Odd-numbered lanes contain extracts from fresh-frozen bark. Even-numbered lanes contain extracts from ethylene-treated bark. Note intraspecific similarity but intraspecific dissimilarity of ethylene induced chitinase isoforms in American chestnut (lanes 2, 6, 10) and in Chinese chestnut (lanes 4, 8, 12).

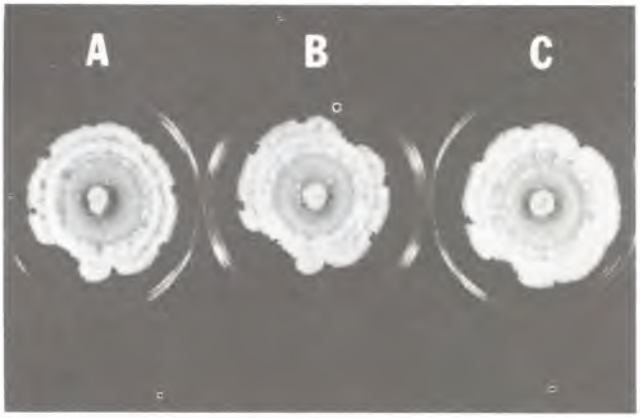

Figure 4. Bioassay of protein extracts from American and Chinese chestnut bark on mycelial growth of *Cryphonectria parasitica*. Wells surrounding fungal colonies growing on potato-dextrose agar amended with methionine and biotin (21) contained extract from 40 mg of bark of trees harvested on A. 12/1/89; B. 3/9/90; and C. 6/22/90. Wells 1–4, clockwise from the top, contained extracts from American chestnut that were: 1. fresh frozen; 2. ethylene treated (ca. 10 ppm); 3. fresh frozen, boiled; and, 4. ethylene treated, boiled. Wells 5–8 contained extracts from Chinese chestnut in the same sequence. Note inhibition of fungal growth by extracts from ethylene-treated Chinese chestnut in A, B, and C and from ethylene-treated American chestnut bark in B.

The present results provide only circumstantial evidence that those hydrolases are active against the chestnut blight fungus, i.e., protein extracts containing those enzymes inhibited mycelial growth and lysed hyphae. Other components of these protein extracts also may be active against the pathogen. To determine this, these enzymes will need to be purified and their effect, singly and in combination, determined. These hydrolases were shown to act synergistically to inhibit some fungi that are in the same Class as *C. parasitica* (17).

Antifungal hydrolases have been implicated in the resistance of some plants to fungal pathogens (2, 7). Additional investigations are required to determine if their induction plays a definitive role in the resistance of Chinese chestnut to blight.

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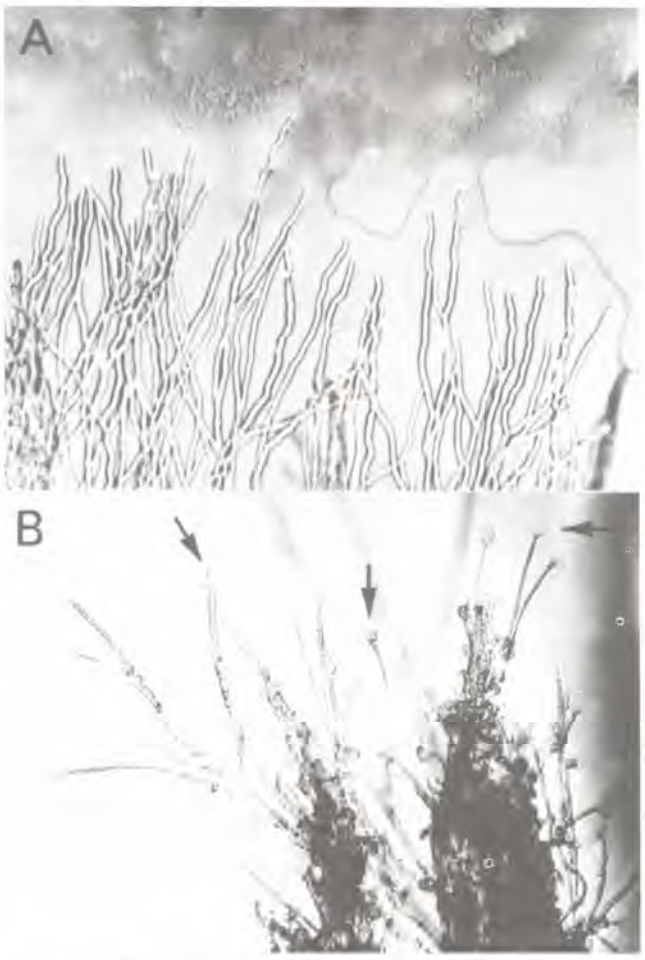

Figure 5. Photomicrographs (ca. 150x) of hyphae of *Cryphonectria parasitica* growing toward wells in potato dextrose agar amended with methionine and biotin (21) containing protein extract from 40 mg of Chinese chestnut bark. A. Hyphae approaching well containing extract of ethylene treated, boiled bark. B. Hyphae approaching well containing extract of ethylene treated bark. Arrows point to lysed hyphae.

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