Search for Novel Peroxidases in Chestnut That Result From the Interaction of Trees With the Chestnut Blight Fungus

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ABSTRACT. The Connecticut Agricultural Experiment Station has an excellent collection of species and hybrids of *Castanea,* planted from 1929 to 1992. These have been used for evaluation of resistance by inoculation with standard virulent strains of *Cryphonectria parasitica.* Peroxidase isozyme patterns form many of the trees have been compared to search for differences that might explain the differences in the reactions of the trees to chestnut blight infection.

There is an excellent collection of hybrids of *Castanea* spp., that were planted between 1929 to 1992 at The Connecticut Agricultural Experiment Station, in New Haven, Conn. These have been used for evaluation of resistance by inoculating with standard virulent strains of *Cryphonectria parasitica* (Murr.) Barr (3), and for a study of chestnut tannins (4). The Experiment Station also has an extensive collection of strains of the blight fungus (1). Few studies have been conducted on the interactions of the pathogen and chestnut species that possess different levels of resistance.

Lignification has been suggested as a mechanism for plant resistance to pathogens, and this subject was reviewed by Vance et al. (16). Early workers reported that when chestnut trees were infected by *C. parasitica,* the phloem reacted by rapidly producing wound periderm, suberizing and forming a lignified barrier (6, 11). In resistant chestnut trees, this effectively walls off the fungus and prevents canker expansion, but in susceptible trees the barriers are breached or circumvented by the fungus. It is not clear whether the rate, extent, or composition of these physical barriers are responsible for the success of the resistant trees, or whether other factors (chemical) are involved. Hebard examined early canker formation in resistant and susceptible trees and found no clear differences in the initial tree reaction (10). More cytological studies are needed on expanding, established cankers on susceptible trees.

The enzyme peroxidase is crucial to lignin formation (8, 9), and Hebard reported strong staining for peroxidase activity in lignifying phloem cells of chestnut during early canker formation (10). Many isozymes of peroxidase have been detected in a number of plant tissues (7). Bal and Linthorst (5) divide them into cationic, vacuolar isozymes with isoelectric points (pI) from 8.1 to 11, and cell-wall-associated, anionic isozymes (pI 4.5-5.6 and pI 3.5-4.0). The latter are thought to function in suberization and lignification. Other roles of peroxidases in disease resistance were examined by Kerby and Somerville (12) who reported an increase in the pI 5.2 and the pI 8.5 isozymes in barley after inoculation with powdery mildew. No difference was seen between resistant and susceptible cultivars, which suggests that these peroxidases were not related to the gene-for-gene resistance in barley. However, the increases may have been associated with the production of appressoria or infection pegs by the fungus, a wound response of the sort expected in chestnut bark infected with chestnut blight.

There is still much to be done to clarify the mechanisms involved in lignification (13). Work is needed on the formation of the complex polymers from a mixture of phenolic alcohols, and the binding between polymers and carbohydrates. The actions of isozymes of peroxidase are thought to play an important role in these processes (8, 9). If the isozymes have different specificities and distributions, the characteristics of the lignin produced could vary according to the isozymes present, and the amount of lignification could be controlled by shifts in isozyme composition.

Three anionic isozymes of peroxidase from chestnut bark-scrapings were described by Santamour et al. (15). Consistent (and different) patterns for *C. dentata* (Marsh.) Borkh. and *C. crenata* Sieb. and Zucc. and vari-

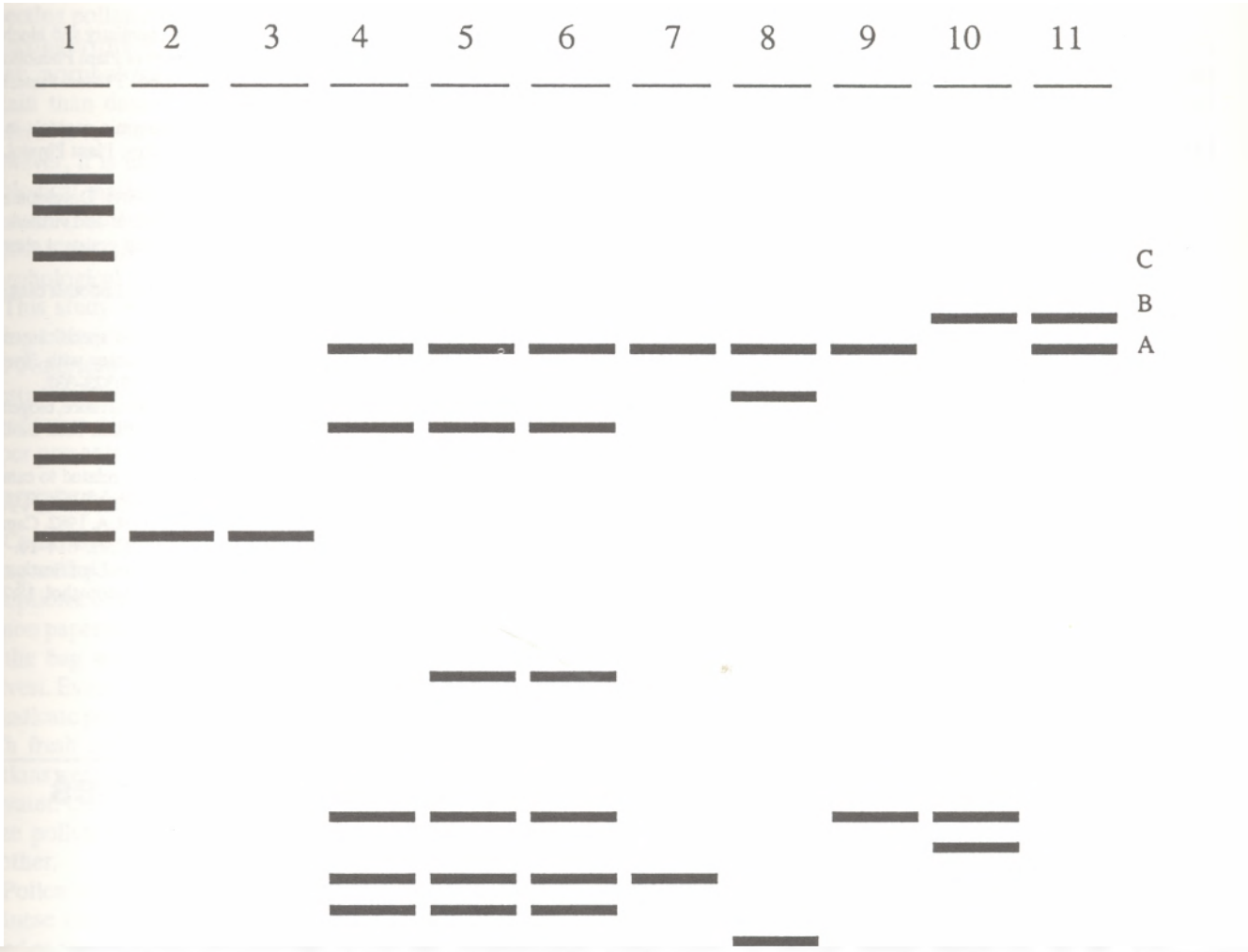

Figure 1. Diagram of the anionic isozymes of peroxidase from dormant-bud and bark sample extracts of chestnut trees (lanes 4 through 11), virulent strains of the blight fungus (lanes 2 and 3), and a horseradish peroxidase control (lane 1). Electrophoresis was on native polyacrylimide gel for seven hours at 30 mA in a pH 8.9 Tris-glycine buffer, stained with diaminobenzidine and peroxide at pH 6. The positions of isozymes A and B reported by Santamour are noted, as well as the relative position of his isozyme C. Extracts from American chestnut trees are in lanes 4, 7, 8, and 9. Lanes 5 and 6 are extracts of samples taken from the tree in lane 4, just beyond the edge of active cankers incited by blight fungus strains 389 (lane 5) and 155 (lane 6). Lanes 7 and 8 are extracts from trees in Watertown, N.Y. reported by F. Ashworth to be slightly resistant to blight infection. The extract in lane 10 is from one of our standard Japanese chestnut trees (R7T7 at the Plantation) and lane 11 is from the Chinese tree 'Mahogany' (R1T15 at the Plantation).

able patterns for C. *mollissima* Bl. were related to their graft compatibility (14, 15).

Our present method for studying peroxidase isozymes of chestnut is to place tissue (twigs or bark pieces) directly into liquid nitrogen. The frozen samples are freeze-dried and ground in a Wiley mill, and extracted with 1% ascorbic acid (sodium), 5 mM potassium metabisulfite, 10 mM cysteine, and 1% Tween 80, adjusted to pH 7.0. The tissue to extracting-solution ratio that has worked best is 1 gm of ground (thy) tissue to 10 ml of solution.

I have confirmed the results of Santamour using extracts from dormant buds in native polyacrylimide gels, electrophoresed vertically in a Tris-glycine buffer at pH 8.9 (2). The gels were stained by allowing them to react with hydrogen peroxide (0.5 ml of a 3% solution in 50 ml) and 3-3'-diaminobenzidine (0.25 mg in 50 ml) in 0.2 M MES buffer, pH 6.0. (Figure 1). American extracts always have the "A" isozyme but not the "B" isozyme, Japanese chestnut extracts always have the "B" and never "A," and Chinese chestnut can have "A," "B," or "AB" (Santamour reported a third isozyme, "C," which we have found in only one tree).

E. Havir and I have used isoelectric focusing gels to partially define the pI relationships of these isozymes. Preliminary results suggest that the pI of isozyme "A" is about 4, and that of "B" is about 5.

Many other peroxidase isozyme bands, in addition to the ones described above, can be detected upon electrophoresis of chestnut twig extracts. Their functions and roles are unknown. We can now compare the isozyme patterns of trees resistant and susceptible to chestnut blight, with mechanical wounds or wounds that have been inoculated with the blight fungus. One such unusual isozyme, which migrates in gels more rapidly than the "A" isozyme, was identified in bark of American chestnut just beyond cankers formed by virulent strains 389 and 155

(Figure 1). Additional studies are in progress to further characterize this isozyme, and look for others that might be involved in the host-pathogen interaction.

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