Plant Physiol. (1976) 57, 920-922

Soluble and Cell Wall Peroxidases in Reed Canarygrass in Relation to Disease Resistance and Localized Lignin Formation[1]

Received for publication November 21, 1975 and in revised form February 27, 1976

CARROLL P. VANCE, JAMES O. ANDERSON,[2] AND ROBERT T. SHERWOOD
United States Regional Pasture Research Laboratory, Agricultural Research Service, United States Department of Agriculture, University Park, Pennsylvania 16802

ABSTRACT

The relationship of peroxidases to an inducible disease-resistance mechanism involving lignification of leaf epidermal cell walls was studied. Reed canarygrass (*Phalaris arundinacea* L.) leaf discs were inoculated with *Helminthosporium avenae* Eidam and floated on water. In inoculated discs, the activity of soluble, ionic wall-bound and covalent wall-bound peroxidases was about twice the level of activity in noninoculated discs. The increase was attributable to increases in activity of three cathodic isoperoxidases and to the appearance of a new cathodic isoperoxidase. Peroxidase activity in cryostat microtome sections of inoculated discs was histochemically localized in the wall near the site of attempted penetration. When inoculated discs were floated on solutions of cycloheximide (25 μg/ml), increases in peroxidase activity were inhibited, and the fungus penetrated the tissue. The inhibition of peroxidase activity was related to inhibition of cathodic isoperoxidase activity. Anodic isoperoxidase activity did not show changes in response to inoculation or cycloheximide treatment.

It was suggested that the resistance mechanism in *P. arundinacea* involves an induction of cathodic isoperoxidases in challenged tissue. These peroxidases may function in the biosynthesis of lignin at the site of attempted penetration.

Plant peroxidases have been extensively investigated with regard to their role in resistance to fungal, viral, and bacterial diseases (14, 15). Peroxidases have also been strongly implicated in lignin biosynthesis (5, 13). Recent studies have implicated lignin formation in the cell walls of reed canarygrass and wheat as a resistance response (11, 12, 16, 17). Vance and Sherwood (17) showed that when the resistance response in reed canarygrass was inhibited by cycloheximide, lignin biosynthesis and activity of soluble enzymes (including peroxidase) involved in lignin biosynthesis were also inhibited. Since the cell wall provides the initial interface between the pathogen and the host, cell wall components such as lignins and peroxidases may be closely involved in the plant's response to a pathogen.

In this study, we have investigated soluble and cell wall peroxidases of reed canarygrass. We have also investigated the localization of peroxidases around the attempted penetration site. The results are discussed in relation to lignin formation and disease resistance in reed canarygrass.

[1] Contribution No. 378, United States Regional Pasture Research Laboratory, Agricultural Research Service, United States Department of Agriculture. Research supported by a cooperative agreement program between the United States Department of Agriculture and The Pennsylvania State University.

[2] Present address: W. Alton Jones Cell Science Center, Lake Placid, NY 12946.

MATERIALS AND METHODS

Reed canarygrass (*Phalaris arundinacea* L.) clone 6049 (9) was grown in a glasshouse bed of peatmoss-vermiculite. Leaf discs (8-mm diameter) were cut with a corkborer from unblemished, expanded leaves. The discs were floated on distilled H_2O or aqueous solutions of cycloheximide (25 μg/ml) in Petri dishes. The upper surface of the disc was thoroughly sprayed with a suspension of *Helminthosporium avenae* Eidam spores or a 0.05-ml drop of spore suspension was pipetted directly onto the disc. The preparation of inoculum was described previously (16).

Cell-free extracts were obtained from samples collected 18 hr after inoculation. The samples were freeze dried. Cell wall and protoplast isoperoxidases were prepared as described by Birecka and Miller (1). Attempts to isolate free peroxidase from cell walls by vacuum infiltration were not successful. Protoplast and free wall peroxidases were isolated together as the soluble peroxidases. The assay for peroxidase was that described by Jennings *et al.* (6). Polyacrylamide disc gel electrophoresis as described by Davis (4) was used to separate anodic and cathodic isoperoxidases. The same amount of protein (400 μg) was applied to all tubes. Either 50 mM benzidine, 25 mM guaiacol, 25 mM *o*-dianisidine, or 25 mM pyrogallol was used as hydrogen donors to visualize isozyme bands. Gels were stained for 30 min, then 0.05% H_2O_2 was added to initiate the reaction.

Protein concentrations were determined by the method of Lowry *et al.* (7).

The histochemical localization of peroxidase was studied in 30-μm thick longitudinal sections of leaf discs cut with a cryostat microtome. Each section was assayed at 24 ± 2 C immediately after cutting. The section was placed on a microscope slide and covered with 2 drops of 50 mM pyrogallol solution (aq.) for 10 min. Then, 2 drops of 0.06% H_2O_2 were added. After 3 min, the solution was blotted away, and the section was rinsed with H_2O. The sections were observed at × 125 to 700 and photomicrographed on Ektachrome EHB 135 film immediately. Three controls were used: (*a*) pyrogallol omitted; (*b*) H_2O_2 omitted; and (*c*) inhibitor of catalase added. In the inhibitor controls, the pyrogallol solution was rinsed off with H_2O before 2 drops of inhibitor (1 mM NaCN or 1 M $NaNO_3$) were added for 2 min. The inhibitor was rinsed off with H_2O, and H_2O_2 solution was added. Assays were run on two or more discs in each of three trials.

RESULTS AND DISCUSSION

Soluble and Cell Wall Peroxidases. Peroxidase activity in soluble fractions and ionic wall-bound fractions increased up to 2-fold in reed canarygrass tissue that was inoculated and incubated on water as compared to tissue that was not inoculated (Table I). The covalent wall-bound peroxidase showed an increase in relative activity in tissue inoculated and incubated on

Table I. *Peroxidase Activity in Reed Canarygrass Leaf Disc Fractions after Various Treatments*

Leaf discs were inoculated with a spore suspension of *Helminthosporium avenae* as described earlier (16). The discs were floated on distilled water or cycloheximide solutions (25 μg/ml) for 18 hr before assay. All values are the mean of three experiments with three replications in each experiment.

Fraction	Treatment	Peroxidase activities		
		ΔA/min/g dry wt	mg protein/g dry wt	ΔA/min/mg protein
Soluble	Inoculated on water	221	65.25	3.38
	Not inoculated on water	101	73.25	1.37
	Inoculated on cycloheximide	71	69.20	1.02
Ionic wall bound	Inoculated on water	77	24.60	3.13
	Not inoculated on water	47	25.20	1.80
	Inoculated on cycloheximide	24	25.50	0.94
Covalent wall bound	Inoculated on water	14.9	. . .[1]	. . .[1]
	Not inoculated on water	11.6	. . .	. . .
	Inoculated on cycloheximide	6.5	. . .	. . .

[1] Covalent protein could not be calculated because of cellulase and pectinase added to the preparation.

water; specific activity could not be determined because of the interference of cellulase and pectinase added during isolation. About 70% of total peroxidase activity was located in the soluble fraction, while the ionic and covalent wall-bound fractions contained 24% and 6%, respectively. The increases observed in peroxidase activity in the inoculated tissue were inhibited by cycloheximide. The activity in cycloheximide-treated tissue was also reduced compared to the noninoculated controls.

Disc gel electrophoresis indicated that there were at least 10 isozymes of peroxidase in soluble and ionic wall-bound fractions of reed canarygrass tissue inoculated and incubated on H_2O (Fig. 1). Four cathodic and six anodic isozyme bands were observed with pyrogallol, guaiacol, and *o*-dianisidine. Isoperoxidase bands C2, C3, C4, and A3 were most prominent. Isoperoxidases C2, C3, and C4 showed much higher relative activity in inoculated tissue than in noninoculated tissue. In cycloheximide-treated tissues, isozymes C2, C3, and C4 showed little relative activity in the soluble fraction and could not be detected in the ionic wall-bound fraction. Isozyme C1 was present in inoculated tissue but could not be detected in cycloheximide-treated or noninoculated tissue. There was no detectable qualitative or quantitative difference in any given anodic isozyme among treatments.

In the covalent wall-bound fractions, isozymes C1, C2, C3, and C4 occurred in the inoculated tissue. Isozyme C1 was not detected in noninoculated or cycloheximide-treated tissue. There was no detectable peroxidase activity in the cathodic portion of the gel from cycloheximide-treated tissue. Anodic covalent wall-bound isoperoxidases showed only bands A2, A3, and A6. Isozymes A1, A4, and A5 were not detected in covalent wall-bound fractions.

The measurements of peroxidase in mycelial extracts of pure cultures of *H. avenae* indicated that the fungus produced very little peroxidase (0.0001–0.005 ΔA/min·mg protein). Thus, it appeared that fungal peroxidase contributed little, if any, to the increased activity in inoculated tissues.

Our results confirm earlier indications that peroxidase activity increases in reed canarygrass in response to attempted penetration by fungi (17). We found both quantitative and qualitative changes in isoperoxidase patterns upon inoculation. The new isozyme may have been of plant origin, since almost no peroxidase activity was detected from mycelial mats of *Helminthosporium avenae*. Treatment with cycloheximide inhibited the observed increases in enzyme activity and alterations in isoperoxidase patterns.

The data indicate that the cathodic isoperoxidases are the isozymes that are affected by attempted penetration. Inhibition of these isoperoxidases with cycloheximide is associated with the tissue becoming susceptible. Cathodic isozymes of peroxidase in other plants show increases in response to injury or disease. Cathodic isozymes of corn (*Zea mays*) show increases in response to cutting injury and fungal infection (2). In Japanese radish (*Raphanus sativus*), a cathodic isozyme has been suggested as being the primary isozyme involved in regulation of lignin formation in response to disease (10). Our findings agree with these observations.

Histochemistry of Cell Wall Peroxidases. Histochemical tests of about 30 freeze-microtome sections from six discs taken from three experiments revealed a pyrogallol-oxidizing enzyme at the penetration site and in the adjacent outer epidermal wall within a radius of about 30 μm. The penetration site and associated wall area stained medium to bright amber when incubated with pyrogallol and H_2O_2 (Fig. 2A). The outer epidermal wall further away was not stained. In control sections incubated with pyrogallol alone (Fig. 2B) or H_2O_2 alone (Fig. 2C), the penetration area was light yellow as a result of a naturally occurring pigment (12). The adjacent epidermal wall was unstained. These results indicated that one or more peroxidases were active in the plant at the pentration site and in the nearby wall. The development of amber color at the penetration site and in the adjacent epidermal cell walls was inhibited by 1 mM NaCN, but was only slightly inhibited by 1 M $NaNO_3$. These data give further evidence that a peroxidase was active at the penetration site (8). The tests also revealed peroxidase activity in guard cells and somewhat less activity in the prickle-hairs, veins, and mesophyll of normal and inoculated discs. Sections of leaves floated on cycloheximide solutions (25 μg/ml) and inoculated with *H. avenae* for 24 hr showed fungal penetration but did not show peroxidase activity at the point of penetration or in the epidermal wall around the site of penetration.

These data indicate that there was a localization of peroxidase activity in the cell wall area around the penetration site. Cycloheximide inhibited localization of peroxidase activity around the penetration site and allowed the fungus to penetrate the tissue. Earlier studies from our laboratory indicated that reed canarygrass produces a localized lignified papilla in the epidermal cell wall at the attempted penetration site (12) and fungi were not able to penetrate the epidermis (16). Treatment with cycloheximide inhibited the formation of these lignified papillae, and as a result, fungi not normally pathogenic to reed canarygrass could penetrate and ramify through the tissue (16). Cycloheximide

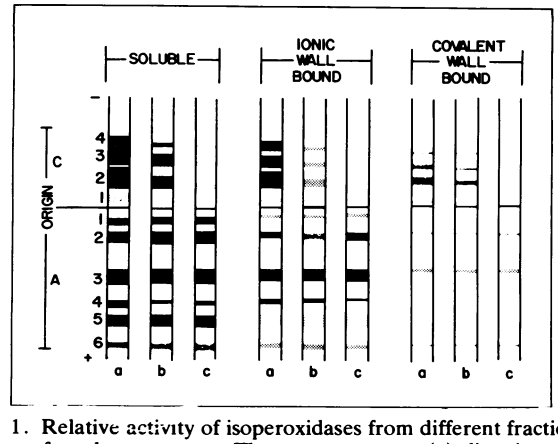

FIG. 1. Relative activity of isoperoxidases from different fractions of leaf discs of reed canarygrass. The treatments are (a) discs inoculated with *Helminthosporium avenae* and incubated on H_2O, (b) discs noninoculated and incubated on H_2O, (c) discs inoculated with *H. avenae* and incubated on cycloheximide (25 μg/ml). Isozyme bands were stained with either 50 mM pyrogallol, 25 mM guaiacol, or 25 mM *o*-dianisidine. The reactions were initiated by adding 0.05% H_2O_2.

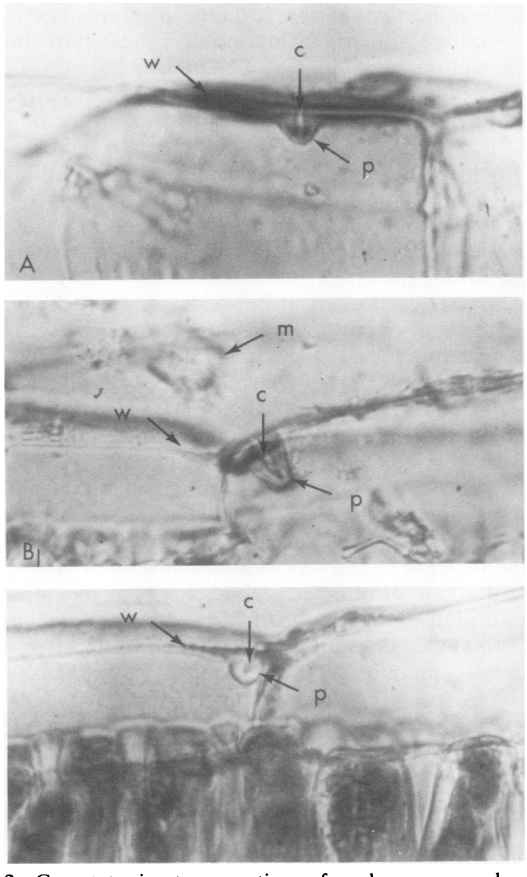

FIG. 2. Cryostat microtome sections of reed canarygrass leaves inoculated with *Helminthosporium avenae*. The leaves show prominent appositional growths (papillae = p) of the outer epidermal walls (w) surrounding the penetration channels (c) of the fungus. The fungal mycelium (m) was torn away during sectioning. A. Incubated with pyrogallol followed by H_2O_2. Deep amber color developed in the papilla and the surrounding 60-μm diameter area of the outer epidermal wall indicative of peroxidase activity localized in these areas. Walls farther away were not heavily stained. B. Control incubated with pyrogallol alone. C. Control incubated with H_2O_2 alone.

also inhibited enzymes associated with lignin biosynthesis (17). Results presented in this study in conjunction with our earlier studies suggest that increased peroxidase activity and altered isozyme patterns in reed canarygrass may be the response of the plant to attempted penetration of fungi. These observed alterations may be involved in the formation of lignified cell wall structures at the site of attempted penetration.

Birecka *et al.* (2, 3) have suggested that increased peroxidase activity in corn and tobacco (*Nicotiana tabacum*) is a nonspecific response to injury; however, Ohguchi and Asada (10) suggested that increased peroxidase activity in Japanese radish is involved in lignin formation which may be involved in resistance.

Data presented in this report are the first showing that inhibition of specific cell wall peroxidases around penetration sites results in susceptibility to nonpathogenic fungi. Studies are now underway to ascertain if inhibition of lignin and peroxidase occurs in a compatible host-pathogen association in reed canarygrass.

Acknowledgments – We would like to thank P. A. Manilla and C. L. Zauzig for technical support.

LITERATURE CITED

1. BIRECKA, H. AND A. MILLER. 1974. Cell wall and protoplast isoperoxidases in relation to injury, indoleacetic acid and ethylene effects. Plant Physiol. 53: 569-574.
2. BIRECKA, H., J. L. CATALFAMO, AND M. O. GARRAWAY. 1975. Cell wall and protoplast isoperoxidases of corn leaves in relation to cut injury and infection with *Helminthosporium maydis*. Plant Physiol. 55: 607-610.
3. BIRECKA, H., J. L. CATALFAMO, AND P. URBAN. 1975. Cell wall and protoplast isoperoxidases in tobacco plants in relation to mechanical injury and infection with tobacco mosaic virus. Plant Physiol. 55: 611-619.
4. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404-427.
5. GRISEBACH, H. AND K. HAHLBROCK. 1974. Enzymology and regulation of flavonoid and lignin biosynthesis in plants and plant cell suspension cultures. *In*: V. C. Runeckles and E. E. Conn, eds., Metabolism and Regulation of Secondary Plant Products. Recent Advances in Phytochemistry, Vol. 8. Academic Press, New York. pp. 21-52.
6. JENNINGS, P. H., B. L. BRANNAMAN, AND F. P. ZSCHEILE, JR. 1969. Peroxidase and polyphenoloxidase activity associated with *Helminthosporium* leaf spot of maize. Phytopathology 59: 963-967.
7. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
8. MAEHLY, A. C. AND B. CHANCE. 1954. The assay of catalases and peroxidases. *In*: D. Glick, ed., Methods of Biochemical Analysis, Vol. I. Wiley-Interscience, New York. pp. 357-424.
9. MARTEN, G. C., R. F. BARNES, A. B. SIMONS, AND F. J. WOODLING. 1973. Alkaloids and palatability of *Phalaris arundinacea* L. grown in diverse environments. Agron. J. 65: 199-201.
10. OHGUCHI, T. AND Y. ASADA. 1975. Dehydrogenation polymerization products of *p*-hydroxycinnamyl alcohols by isoperoxidases obtained from downy mildew-infected roots of Japanese radish (*Raphanus sativus*). Physiol. Plant Pathol. 5: 183-192.
11. RIDE, J. P. 1975. Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. Physiol. Plant Pathol. 5: 125-134.
12. SHERWOOD, R. T. AND C. P. VANCE. 1976. Histochemistry of papillae formed in reed canarygrass leaves in response to noninfecting pathogenic fungi. Phytopathology 66: 503-510.
13. STAFFORD, H. A. 1974. Possible multienzyme complexes regulating the formation of C_6-C_3 phenolic compounds and lignins in higher plants. *In*: V. C. Runeckles and E. E. Conn, eds., Metabolism and Regulation of Secondary Plant Products. Recent Advances in Phytochemistry, Vol. 8. Academic Press, New York. pp. 53-79.
14. STAHMANN, M. A. 1967. Influence of host-parasite interactions on proteins, enzymes and resistance. *In*: C. J. Mirocha and I. Uritani, eds., The Dynamic Role of Molecular Constituents in Plant Parasite Interaction. Bruce Publishing Co., St. Paul, Minn. pp. 357-372.
15. STAHMANN, M. A. AND D. M. DEMOREST. 1973. Changes in enzymes of host and pathogen with special reference to peroxidase interaction. *In*: R. J. W. Byrde and C. V. Cutting, eds., Fungal Pathogenicity and the Plant's Response. Academic Press, New York. pp. 405-422.
16. VANCE, C. P. AND R. T. SHERWOOD. 1976. Cycloheximide treatments implicate papilla formation in resistance of reed canarygrass to fungi. Phytopathology 66: 498-502.
17. VANCE, C. P. AND R. T. SHERWOOD. 1976. Regulation of lignin formation in reed canarygrass in relation to disease resistance. Plant Physiol. 58: 915-919.