Pergamon

PII: S0031-9422(97)01021-2

Phytochemistry, Vol. 48, No. 1, pp. 41–47, 1998
© 1998 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0031-9422/98 $19.00+0.00

SEASONAL VARIATION OF PEROXIDASE ACTIVITY IN CHESTNUT TREES

EVELYN A. HAVIR,* and SANDRA L. ANAGNOSTAKIS

Departments of Biochemistry and Genetics (E.A.H.) and Plant Pathology and Ecology (S.L.A.), Connecticut Agricultural Experiment Station, Box 1106, New Haven, CT 06504, U.S.A.

(*Received* 15 *July* 1997; *in revised form* 3 *October* 1997)

Key Word Index—*Castanea dentata*; *C. mollissima*; *C. crenata*; chestnut; peroxidase; seasonal variation; blight resistance.

Abstract—Peroxidase (E.C.1.11.1.7) activity in twigs of American (*Castanea dentata*), Chinese (*C. mollissima*), and Japanese (*C. crenata*) chestnut was determined monthly over an eight month period. The total amount of peroxidase activity was highest in December and April for most trees. An inhibitor of peroxidase activity was present in the extracts which could be removed by gel filtration. Extracts from healthy twigs had consistent major bands on IEF gels. However, a new band was detected in bark of American chestnut after wounding or infection with chestnut blight fungus (*Cryphonectria parasitica*). © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The American chestnut (*Castanea dentata*) was once a dominant hardwood in the eastern U.S. forest. The introduction of the chestnut blight fungus (*Cryphonectria parasitica*) into the United States in the late 1800's reduced the tree to an understory shrub that becomes infected, dies, sprouts from the base, and grows until infected again [1]. Chinese (*C. mollissima*) and Japanese (*C. crenata*) chestnuts may develop cankers, but the fungus is usually restricted to superficial (bark) growth and cambial tissue is rarely killed. The factors which convey resistance to this wound pathogen in *C. mollissima* and *C. crenata* are not known. Several early studies suggested that bark tannins might be important [2, 3], but a subsequent study revealed no evidence for this [4]. McCarrol and Thor investigated the effect of bark extracts on pectolytic, cellulytic, and proteolytic activities of the fungus and found Chinese chestnut bark extracts to be more effective in inhibiting fungal polygalacturonase than American chestnut bark extracts [5]. Gao and Shain found that a proteinaceous component of Chinese bark, but not American bark, was a highly effective inhibitor of the same enzyme [6].

Peroxidases (E.C.1.11.1.7) in plants have been extensively studied for their role in defense against plant pathogens as well as their involvement in the last step of lignin formation (e.g. [7–9]). Santamour [10, 11] examined the relationship of peroxidases in various *Castanea* species to graft compatibility. Other studies have examined the population genetics of enzymes including peroxidases in chestnut [12–14], but to date no attempt to evaluate the role of peroxidase activity in chestnut blight resistance has been reported.

We first undertook to determine whether the peroxidase levels in American, Chinese and Japanese chestnut were different and what the effect of infection was on the levels of activity. It became evident that the interpretation of the results would depend on factors such as the seasonal variation in enzyme activity, differences among individual trees, and the presence of inhibitors. A study of such factors was undertaken and reported here as well as the results of the initially proposed study.

RESULTS AND DISCUSSION

Trees used in this study

The origin of each tree used in this study is given in Table 1. They have been used extensively in the chestnut breeding program at the Connecticut Agricultural Experiment Station. A detailed study of the resistance shown by five of these trees has been published [15]. All the Chinese and Japanese trees show resistance to infection by *Cryphonectria parasitica*. Of the American chestnut trees, A-2 shows greater resistance than

---

*Author to whom correspondence should be addressed. Tel.: 203-789-7231. Fax: 203-789-7232.

Table 1. Descriptions of the trees used in this study. PI numbers are the numbers assigned to imported seed and plants by the Foreign Plant Introduction Office, Bureau of Plant Industry, USDA (now the U.S. National Plant Germplasm System, USDA, ARS). A list of the *Castanea* holdings of the Connecticut Agricultural Experiment Station is available from Anagnostakis

Tree	Species	Description
American		
A-1	*Castanea dentata* (Marshall) Borkhausen	Seedling from Michigan or Wisconsin, planted 1976
A-2	*C. dentata*	"Scientists' Cliffs", graft of a tree in Glendale, MD private community, also called FP #1000, grafted here 1959
A-3–A-7	*C. dentata*	Seedlings from a forest plot in Rocky Hill, CT, planted 1986
Chinese		
C-1	*C. mollissima* Blume	PI #70315, "Mahogany", hardy tree from northeastern China, planted 1929
C-2	*C. mollissima*	PI #104063, "MAU", tall Chinese, Chekiang Prov., China, planted 1935
Japanese		
J-1	*C. crenata* Siebold and Zuccarini	PI #78626, wild tree, Oguriyama, Amori Ken, Japan, planted 1929
J-2	*C. crenata*	PI #104016, "GO", Numakunai Eirinsho, Iwate Ken, Japan, planted 1935

A-1. The other American trees used (Table 1) are open pollinated seedlings of numbered native trees which are part of another study on the biological control of chestnut blight disease.

Peroxidase activity in American chestnut twigs

Total peroxidase activity in twigs collected in May of the seven American chestnut trees (see Table 1) is shown in Fig. 1. Two values are given for each sample, one for the extract, and the other for the extract after gel filtration. Passage through Sephadex always resulted in an increase of activity, but the increase was variable, i.e. from a 19-fold increase for A-3 to a 1.8-fold increase for A-1. In addition, although all samples were collected at the same time and all inhibitor removed by gel filtration, the total peroxidase levels varied at least 6-fold thus making it difficult to determine an "average" peroxidase activity for this species of *Castanea*. In a later section, the investigation of the nature of the inhibition shown by extracts will be discussed in greater detail.

Seasonal variation in peroxidase activity in twigs

For the main part of this study six chestnut trees, two each of American, Chinese and Japanese were chosen (Table 1). The variation in total peroxidase activity in twigs collected and assayed monthly from October to May is shown in Fig. 2, and is that found

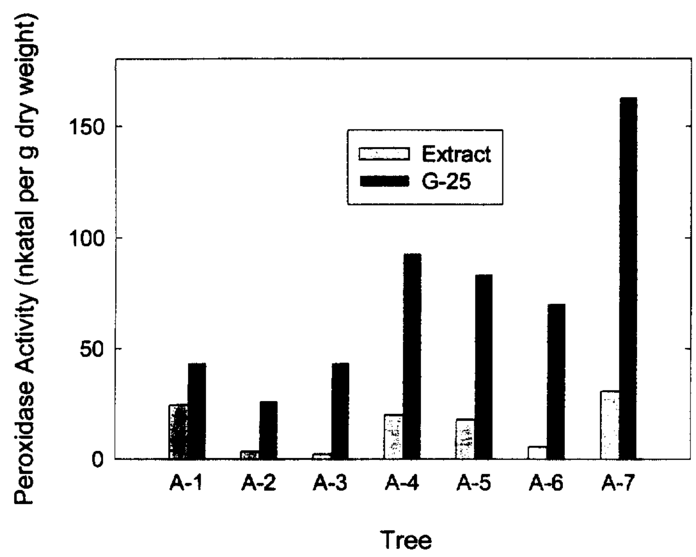

Fig. 1. Total peroxidase activity before and after gel filtration in twigs of American chestnut collected in May.

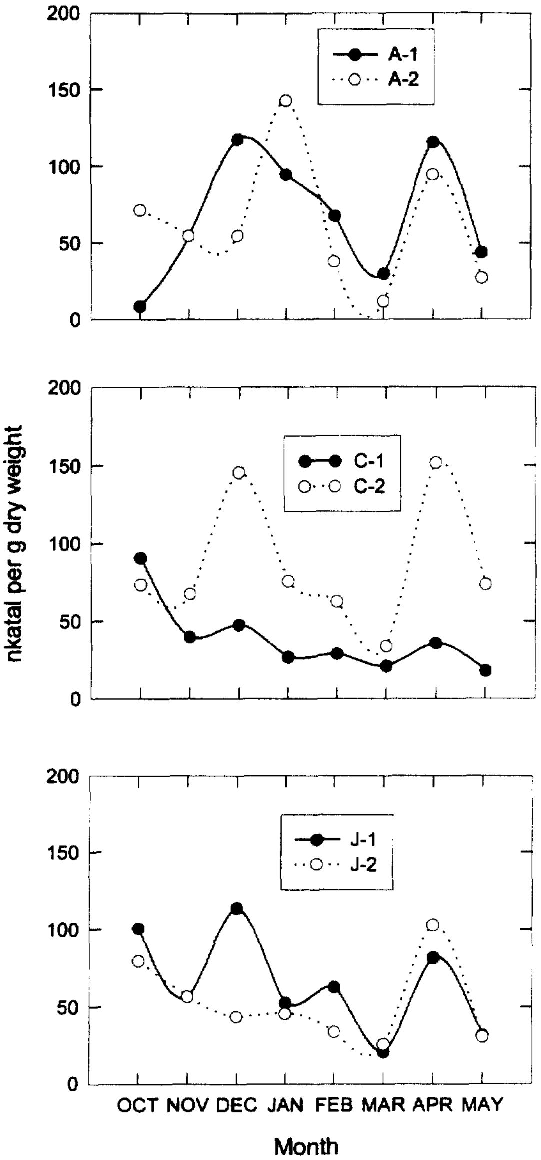

Fig. 2. Seasonal variation in total peroxidase activity after gel filtration in American, Chinese, and Japanese chestnut twigs.

after passage of the extract through Sephadex. In general, A-1, A-2, C-2, and J-1 showed peaks of activity in December or January and in April. C-1, on the other hand showed little or no seasonal variation, and J-2 showed only the April peak of activity. It is interesting that all three species have similar levels of peroxidase at their peak activity (i.e. 100–150 nkatal per gram sample), but it is evident that results could be misleading as to total peroxidase activity if a single tree or one time period is chosen. We cannot conclude that total peroxidase levels are correlated with resistance.

Seasonal variation in peroxidase levels has been reported for a number of woody species. For example, Nelson demonstrated that in walnut, peroxidase levels were highest in the late growth to early dormant period and suggested that the dormant period was the time of heartwood formation for both walnut and cherry [16]. Sagisaka and Asada measured peroxidase activity in crude extracts of poplar xylem tissue and found low activity from June to September, which increased three-fold from October to May [17]. Because there was no purification of the crude extract, it is possible that their results could be explained by the increased presence of inhibitors during the summer. The results of Imberty *et al.* also suggested seasonal fluctuations in the phloem and xylem peroxidase activity [18].

Separation of peroxidases in extracts by isoelectric focusing

When peroxidases from twigs were separated by IEF over a wide range of pH the major bands of peroxidase activity focused below pH 6.0 (data not shown). Therefore the samples were separated by IEF in the pH range of 3.5–5.0 with the results shown in Fig. 3. The major bands of peroxidase activity, labelled 1 to 4, correspond to pH values of approximately 5.0, 4.8, 4.4, and 3.5, respectively. The relative positions of the bands did not change from gel to gel, but the pH profile in the gel sometimes changed with source of ampholytes. Peroxidases-2 and -4 were present in all species. Peroxidase-1 and peroxidase-3 correspond to the B and A bands of Santamour [10] and Anagnostakis [12] which are determined by alleles at a single locus. American and Chinese trees had peroxidase-3, but peroxidase-1 was also present in C-1. Both Japanese trees contained peroxidases-1, -2, and -4. These samples shown in Fig. 3 were collected in May, but each month's samples were examined for distribution of peroxidase activity using IEF.

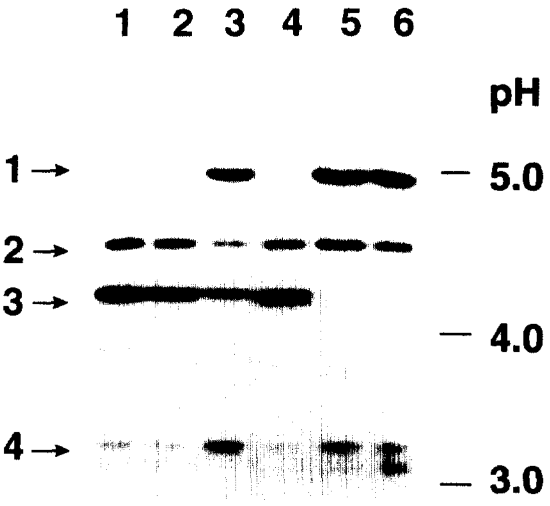

Fig. 3. Separation by IEF of peroxidase from twigs of American, Chinese, and Japanese chestnut trees collected in May. Lanes 1–6 contained extracts of A-1, A-2, C-1, C-2, J-1, and J-2, respectively.

Although the total activity differed from month to month for some trees (see Fig. 2), the major bands remained the same.

Inhibition of peroxidase activity

As noted in Fig. 1, the value for total peroxidase increased greatly when extracts were passed through Sephadex. The increase in peroxidase activity after gel filtration of the extracts obtained from monthly sampling is shown in Fig. 4. No simple pattern of inhibition emerged. Samples from two trees, A-1 and C-2, showed little or no increase for most of the year whereas C-1, J-1, and J-2 most often showed a 10-fold increase. It was not possible to determine visually which extracts would show a large increase in activity.

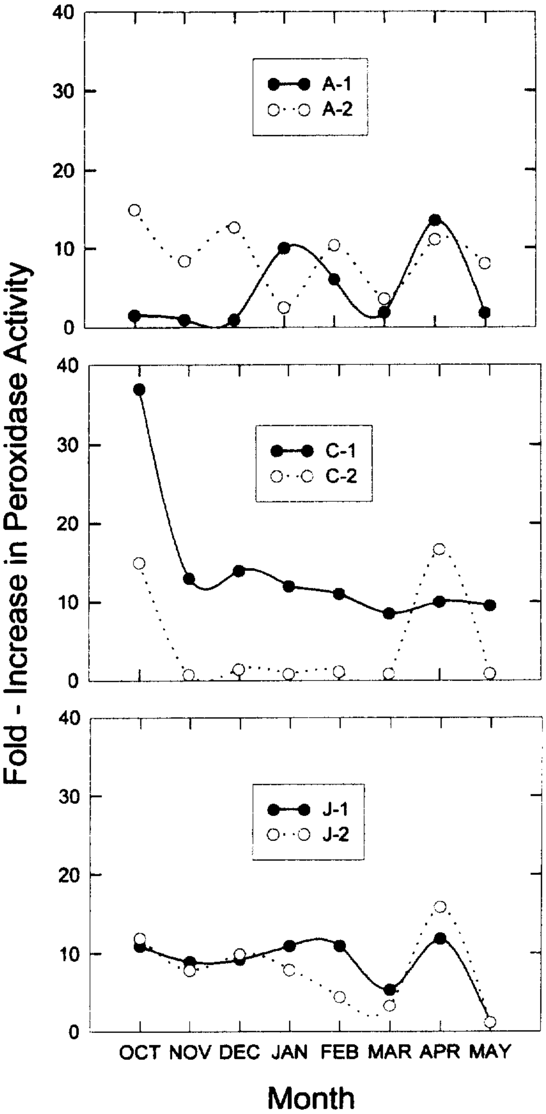

Fig. 4. Increase in peroxidase activity after gel filtration of extracts. Fold-increase is defined as the activity recovered after gel filtration divided by the activity in the extract.

When buffer eluates from the Sephadex columns were added to either chestnut peroxidase after gel filtration or to horseradish peroxidase there was no inhibition. However, fractions eluted with NH_4OH contained large amounts of inhibitor when tested with either chestnut or horseradish peroxidases (data not shown). Thus the inhibitor is probably not a protein or a low molecular weight compound, but has the characteristics indicative of a phenolic compound. Such compounds are known to inhibit many enzymes.

Extraction with $CaCl_2$

It has been shown that a high ionic strength buffer increases the amount of extractable peroxidases from some tissues [19, 20]. When samples of twigs collected in May were extracted with either buffer alone or 0.20 M $CaCl_2$ in buffer and the extracts assayed for peroxidase activity, we obtained the results shown in Table 2. The presence of $CaCl_2$ resulted in a 1.4 to 1.9-fold increase of peroxidase in extracts. However, after gel filtration the increases due to $CaCl_2$ were less, with the exception of the peroxidases from tree C-2 which still showed a 1.9-fold increase. In contrast, when blueberry fruit was extracted with $CaCl_2$, the increases were 2- to 4-fold higher [20]. It is possible that in our experiments the effect of the $CaCl_2$ is due to the removal or binding of inhibitor since the effect of the $CaCl_2$ was much lower after gel filtration, a step which decreased inhibition (see Fig. 1). Adding $CaCl_2$ to the *in vitro* assay did not stimulate peroxidase activity.

Although extraction with $CaCl_2$ increased activity in the extracts it did not significantly alter their phenolic content as shown in Table 2. For two samples, C-1 and J-1, there was a slight decrease in phenolic content, but for others (A-1, A-2, J-2) there was an

Table 2. Effect of $CaCl_2$ on extractable peroxidase activity and total phenol content*

	Peroxidase activity ratio†		Phenol content‡	
Tree	Extract	Gel filtrate	$+CaCl_2$	$-CaCl_2$
A-1	1.4	1.2	240	210
A-2	1.8	1.2	240	240
C-1	1.4	1.0	180	200
C-2	1.9	1.9	100	90
J-1	1.5	1.3	210	270
J-2	1.7	1.1	230	150

*Twigs were collected in May, samples lyophilized, extracted and assayed as described in Experimental. The peroxidase activities in the extract without $CaCl_2$, for samples A-1 through J-2 were 45, 30, 20, 75, 35 and 34 nkatal per gram dry weight, respectively.

†The activity extracted with $CaCl_2$ is divided by the activity extracted without $CaCl_2$.

‡mg/g dry weight.

apparent increase. The results do not rule out the removal of a specific peroxidase inhibitor by $CaCl_2$ which is not reflected in a change in total phenolic content. Analysis by IEF at both pH 3 to 10 and pH 3.5 to 5 did not reveal the presence of any new bands in $CaCl_2$ extracted samples.

It has been suggested that those peroxidases ionically-bound to cell walls which require high salt extraction are active in the last step of lignification and they have a specificity for the substrate syringaldazine [7, 8, 18], although this has been questioned for some plants [21]. We tested this substrate on extracts of trees A-1, C-1, and J-1 prepared with and without $CaCl_2$, and before and after gel filtration. The activity with syringaldazine was 5% or less of the activity with guaiacol in all cases (data not shown). It is possible that other extraction methods are needed for chestnut. Aside from substrate specificity a number of other criteria must be met before assigning a peroxidase a specific role in lignin biosynthesis [9].

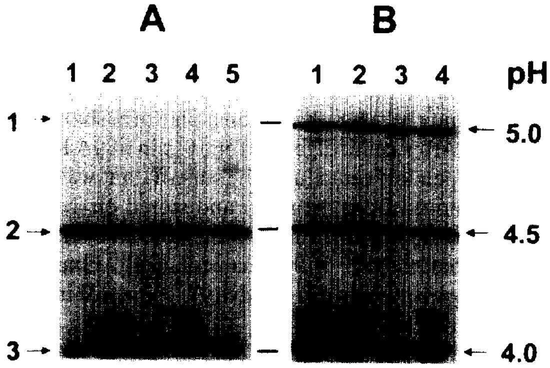

Fig. 5. Separation by IEF of peroxidase at the site of infection or wounding. A: American tree A-1, lanes 1–5, control (non-wounded tissue) canker-1, canker-2, wound-1, wound-2, respectively. B: Chinese tree C-1, lanes 1–4, canker-1, canker-2, wound-2, control (non-wounded tissue) respectively.

Effect of wounding and infection on peroxidase activity

American and Chinese chestnut stems were infected by *C. parasitica*, sampled, and the samples analyzed for total peroxidase activity. The results are shown in Table 3. For A-1 and A-2 peroxidase activity was lowest in the sample nearest the canker, but wounding alone also decreased peroxidase levels. For C-1 and C-2 there was a smaller decrease of peroxidase in canker tissue. Wounding had little (C-1) or no (C-2) affect on peroxidase levels. In the two experiments shown here it would appear that infection by *C. parasitica* could spread more rapidly in American chestnut than in Chinese by inactivating peroxidases which might function in defense.

Two of the samples shown in Table 3 (A-1 and C-1) were analyzed by IEF. In Fig. 5A, non-wounded tissue (lane 1) shows the two major peroxidases found in American chestnut. However, samples of canker-1 and wound-1 (lanes 2 and 4, respectively) show the appearance of a new band of peroxidase just above peroxidase-3. The new band is absent from the samples further from the canker or wound (lanes 3 and 5), and thus appears to be a response to wounding, although it is much more intense in canker-1 than in wound-1. The analysis of the samples by IEF in the pH 3 to 10 range did not reveal any new bands of activity above pH 5. In Fig. 5B a similarly positioned band is present in all samples from the Chinese chestnut, although it is more intense in the infected samples (lanes 1 and 2) than in wounded and non-wounded control (lanes 2 and 4, respectively). Also, in lanes 1 and 2 there is an additional band just below peroxidase-1 which may arise in response to infection. Its position does not correspond to any of the fungal peroxidases (data not shown).

The expression of peroxidase in response to wounding or infection has been described for several plants (e.g. [22, 23]). While the results presented in Fig. 5 suggest that such an expression occurs in chestnut, it is only the first step in exploring the role of peroxidase in the species.

Table 3. Effect of wounding and infection on peroxidase levels in chestnut bark extracts after gel filtration*

Sample†	A-1‡	A-2	C-1	C-2
		Percent of control		
Canker-1	65	30	81	71
Canker-2	82	54	83	93
Wound-1	74	42	81	107
Wound-2	101	78	92	110

* The data shown are based on the means of the duplicate samples from five cankers and three wounds for each species.

† Sampling of wounded and infected bark as described in Experimental.

‡ Control activities for A-1, A-2, C-1, and C-2 were 85, 41, 45, and 59 nkatals per gram dry weight, respectively.

EXPERIMENTAL

Plant material

Three- to five-mm diameter twigs of the current seasons growth (previous season for winter collections) were collected monthly from the trees described in Table 1, and frozen at −20°C. The frozen twigs were lyophilized on a Virtis freeze-dryer until dry, ground in a Wiley mill to a fine powder, and stored at −20°C until assayed. The yield was 1.0 g or more for each sample. Extraction and assay were usually performed within several days of grinding. However duplicate assays on material stored up to several months showed that there was little or no loss of peroxidase activity on storage.

Extraction of tissue and peroxidase assay

Aliquots (150 mg) of ground twig tissue were placed in Eppendorf tubes, 1.0 ml of 0.05 M MES, pH 6.0 added, and the tubes shaken on a platform shaker for 20 min. After centrifugation for 10 min at 14 K rpm in an Eppendorf centrifuge, the supernatant was carefully removed from the pellet and stored on ice until assay. Portions of the supernatant were assayed spectrophotometrically with guaiacol [24]. Duplicate assays agreed within 5%. Activity is expressed in nkatal per gram of dry bark.

Gel filtration

Columns (0.4 × 8.0 cm) of Sephadex G-25 were prepared in Pasteur Pipets and equilibrated with 0.05 M MES, pH 6.0. Extract (0.2 ml) was applied to the column and eluted with the equilibration buffer. Five frs of 0.4 ml each were collected and assayed for peroxidase activity. All of the eluted activity was recovered in frs 3 and 4. For some samples, 10 frs of buffer eluates were collected and followed by a further 6 frs eluted with 0.2 N NH_4OH. These eluates were taken to dryness *in vacuo* and redissolved in equilibration buffer.

Isoelectric focusing

Isoelectric focusing was performed on 1.5 mm gels (10% acrylamide) using the Mighty Small apparatus (Hoefer) according to the procedure of Robertson *et al.* [25]. The ampholytes (Bio-Rad) were either pH 3.5–5.0 or pH 3–10. Ampholytes added to the spectrophotometric assay for peroxidase were not inhibitory. Samples of the extracts (5–10 μl) were mixed with glycerol and ampholytes to final concentrations of 15 and 5%, respectively. After electrophoresis (about 18 h at 350 V maximum) a strip at the edge of the gel (about 1 cm wide) was removed and the remainder of the gel was stained for peroxidase activity using H_2O_2 and 3,3′-diaminobenzidine [12]. The 1 × 6 cm strip of gel was cut into 1 × 0.5 cm segments, which were placed in tubes containing 1.0 ml of demineralized water. The tubes were shaken for 2 to 3 h, and the pH of each sample was measured.

Phenolic content

Total phenolics were measured with Folin–Ciocalteu's phenol reagent using *p*-coumaric acid as standard [26].

Effect of wounding and infection on peroxidase activity

Stems with thin bark were cut from trees A-1 and C-1 in January. The A-1 stems were 1.7 cm to 3.0 cm diameter, and ring counts indicated that they were 5-, 6- and 7-years-old. The C-1 stems were 2.0 to 2.3 cm diameter and 8–9-years old. Eight discs 3 mm diameter were cut from the bark of each species, and five of the wounds were inoculated with plugs of strain 389 of *C. parasitica* (ATCC #38980). The stems were placed in a humid atmosphere at room temperature for two weeks, and cankers formed around the inoculated wounds. In a second experiment equivalently-sized stems of A-2 and C-2 were cut in April, inoculated with strain 155 of *C. parasitica* (ATCC #38755) and incubated as above. Canker tissue, approximately 250–300 mm^2, was taken as sample 1 and canker edge, about 200 mm^2, as sample 2. An equivalent amount of tissue was sampled around the wounds. Non-wounded tissue served as a control for the samples. After freeze-drying, the samples were ground and stored at -20°C until they were extracted and assayed as described above.

Acknowledgements—We wish to thank Pamela Sletten and Carol Clark for skillful technical assistance, and acknowledge partial funding from a USDA McIntire-Stennis grant (SLA).

REFERENCES

1. Anagnostakis, S. L., *Advances in Botanical Research*, 1995, **21**, 125.
2. Nienstaedt, H., *Phytopathology*, 1953, **43**, 32.
3. Hebard, F. V. and Kaufman, P. B., *Proceedings, American Chestnut Symposium*, 1978, p. 63.
4. Anagnostakis, S. L., *Journal Chemical Ecology*, 1992, **18**, 1373.
5. McCarroll, D. R. and Thor, E., *Physiological Plant Pathology*, 1985, **26**, 367.
6. Gao, S. and Shain, L., *Physiological and Molecular Plant Pathology*, 1995, **46**, 199.
7. Harkin, J. M. and Obst, J. R., *Science*, 1973, **180**, 296.
8. Catesson, A.-M., Imberty, A., Goldberg, R. and Czaninski, Y., in *Molecular and Physiological Aspects of Plant Peroxidases*, ed. H. Greppin, C. Penel and Th. Gaspar. University of Geneva, Switzerland, 1986, p. 189.
9. Lewis, N. G. and Yamamoto, E., *Annual Review Plant Physiology and Plant Molecular Biology*, 1990, **41**, 455.
10. Santamour, F. S., Jr., McArdle, A. J. and Jaynes, R. A., *Journal of Environmental Horticulture*, 1986, **4**, 14.
11. Santamour, F. S., Jr., *Ibid*, 1988, **6**, 33.
12. Anagnostakis, S. L., *Hortscience*, 1991, **26**, 1424.
13. Huang, H., Dane, F. and Norton, J. D., *Journal American Society Horticultural Science*, 1994, **119**, 840.
14. Pigliucci, M., Benedettelli, S. and Villani, F., *Canadian Journal of Botany*, 1990, **68**, 1692.
15. Anagnostakis, S. L., *Canadian Journal of Forest Research*, 1992, **22**, 568.
16. Nelson, N. D., *Canadian Journal of Botany*, 1978, **56**, 626.

17. Sagisaka, S. and Asada, M., *Plant Cell Physiology*, 1986, **27**, 1599.
18. Imberty, A., Goldberg, R. and Catesson, A.-M., *Planta*, 1985, **164**, 221.
19. Abeles, F. B. and Biles, C. L., *Plant Physiology*, 1991, **95**, 269.
20. Miesle, T. J., Proctor, A. and Lagrimini, L. M., *Journal American Society Horticultural Science*, 1991, **116**, 827.
21. Church, D. L. and Galston, A. W., *Plant Physiology*, 1988, **88**, 679.
22. Espelie, K. E., Franceschi, V. R. and Kolattukudy, P. E., *Plant Physiology*, 1986, **81**, 487.
23. Hammerschmidt, R., Nuckles, E. and Kúc, J., *Physiological Plant Pathology*, 1982, **20**, 73.
24. Johnson-Flanagan, A. M. and Owens, J. N., *Plant Physiology*, 1985, **79**, 103.
25. Robertson, E. F., Dannelly, H. K., Malloy, P. J. and Reeves, H. C., *Analytical Biochemistry*, 1987, **167**, 290.
26. Glazener, J. A., *Physiological Plant Pathology*, 1982, **20**, 11.