

## Changes in Levels and Isozymes of Peroxidase in Wounded Peach Bark

M. M. GOODIN,<sup>1</sup> A. R. BIGGS,<sup>2</sup> AND A. M. CASTLE<sup>3</sup>

### Abstract

Bark on one-yr-old branches from six peach cultivars was mechanically wounded then examined over time for changes in peroxidase isozymes, total peroxidase activity, and total protein. Four peroxidase isozymes were identified and changes in total activity were associated mostly with one isozyme. Changes in peroxidase activity in response to wounding were observed with activity increasing to the highest levels at 4 and 7 days post-wounding. At 10 and 14 days post-wounding, peroxidase levels had returned to levels similar to the control. An unidentified wound response protein was detected in these experiments. The timing of the appearance of this protein was correlated with the known relative susceptibility of the cultivars to the peach canker pathogens, *Leucostoma persoonii* and *L. cincta*. Comparisons of the peroxidase isozyme profiles and total peroxidase activity among cultivars provided no indication that peroxidase profiles could be used as a marker for resistance to the peach canker pathogens.

### Introduction

Peroxidase are found widely in plants and animals and appear to be ubiquitous in nature (16). The roles of peroxidase in plants are varied and include diverse processes such as regulating fruit development (10) and conferring disease resistance (8). Previous studies (1, 2, 3) have shown that (*Prunus persica* (L.) Batsch) bark responds to wounding by forming a ligno-suberized zone around the wounded area. The rate of suberin production can be correlated to the known relative susceptibility of the peach cultivars to the canker-causing fungi *Leucostoma cincta* and *L. persoonii*. Because of the known role of peroxidase in the lignification and suberization processes, this study was carried out to investigate whether

peach cultivars of differing susceptibility to the canker pathogens differed in their peroxidase activities or in their production of peroxidase isozymes in response to wounding.

### Materials and Methods

#### Bark Samples

Four peach cultivars, 'Redhaven,' 'Earlired,' 'Vanity,' and 'Vivid,' and two clones from Chinese germplasm, V68101 and V68051, were used in this study. Cultivars were selected to represent a range of susceptibility to *Leucostoma* spp. based on historical field performance ratings with visual assessment and a 1 to 10 numerical rating system: 1 = no canker observed; 2 = trace in 1- or 2-year-old wood; 3 = canker very light in major limbs of tree; 4 = canker light to moderate in trunk, crotch, and lower scaffold; 5 = canker moderate in trunk, crotch, and lower scaffold, but severe in minor branches; 6 = canker severe in one of trunk, crotch, or scaffold; 7 = canker severe in two of trunk, crotch, or scaffold; 8 = tree alive but canker likely to become major cause of death, disease severe in trunk, crotch, and scaffold; 9 = tree dying, canker severe and judged to be the major cause of dying; 10 = tree dead, canker severe and judged to be the major cause of death (R. E. C. Layne, personal communication). The order of cultivars and clones from resistant to susceptible was determined to be: V68101, V68051, 'Redhaven,' 'Vanity,' 'Vivid,' and 'Earlired.' One-year-old branches from four five-year-old trees of each cultivar were

<sup>1</sup>Department of Plant Pathology, The Pennsylvania State University, University Park, PA 16802.

<sup>2</sup>Division of Plant and Soil Sciences, West Virginia University, University Experiment Farm, P. O. Box 609, Kearneysville, WV 25430.

<sup>3</sup>Department of Biology, Brock University, St. Catharines, Ontario, Canada L2S 3A1.

mechanically wounded, five wounds per branch, with a No. 2 cork borer (4 mm diam). For sampling, a circle of bark was removed from around the wound at 0, 4 h, and 1, 2, 4, 7, 10 and 14 days post-wounding with a No. 5 cork borer (10 mm diam) and stored on ice for 2-4 h and lyophilized. Bark samples were lyophilized overnight and then stored at -20 C. The tissue pieces were bulked at each collection time and thus one sample consisted of twenty tissue pieces from each cultivar at each time. The experiment was repeated twice over the periods of June 26 to July 10 and July 24 to Aug. 7, 1989.

#### Enzyme Extraction and Protein Determination

Total soluble proteins were collected by first grinding the bark samples in liquid nitrogen after which 3 ml of extraction buffer (0.01 M phosphate buffer pH 6.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1  $\mu$ M phenylmethyl-sulphonylfluoride (PMSF), 1  $\mu$ M iodoacetamide, and 1% polyvinyl pyrrolidone were added to the powdered bark and collected in 30  $\mu$ l corex centrifuge tubes. The tissue homogenate was vortexed twice for 15 sec and then centrifuged at 10,000  $\times$  g for 10 min. The supernatant was collected and lyophilized until dry. The lyophilized supernatant was resuspended in 0.01 M phosphate buffer (pH 6.0) and the mixture adjusted to 20% glycerol. Fifty  $\mu$ l aliquots of the crude extract were quick frozen in liquid nitrogen and stored at -70 C. Crude extract prepared in this way was used for electrophoresis and spectrophotometric assays without further purification. The amount of protein in the extracts was determined with a Bio-Rad (Richmond, CA) protein assay with bovine serum albumin (BSA) as a standard. The method followed for the assay was that supplied by the manufacturer.

#### Gel Electrophoresis

Equivalent amounts of protein (15  $\mu$ g) were separated by 0.75 mm on

10% polyacrylamide slab gels which had 4% stacking gels. Gels were run in Tris-glycine buffer with a Bio-Rad mini-Protean II electrophoresis cell. Instructions for gel preparation and run conditions were those supplied by the manufacturer. After electrophoresis, gels were stained for peroxidase activity with guaiacol-coomassie blue double stain by following the procedure of Shimoni and Reuveni (14).

#### Spectrophotometric Analysis

Peroxidase activity was assayed spectrophotometrically with guaiacol (o-methoxy phenol) as a substrate (13). Equivalent amounts of protein (15  $\mu$ g) were added to 1.5 ml microcentrifuge tubes containing 1.0 mL of guaiacol (1% solution in 0.01 M phosphate buffer pH 6.0 and 50  $\mu$ l 30% hydrogen peroxide. Absorbence at 420 nm was read with a Beckman DU-50 spectrophotometer after a 5 min incubation at room temperature (about 22 C). Factorial analysis of variance (cultivar, time post-wounding and experiment) was used to examine the data (ANOVA procedure, SAS Institute, Cary, NC). Spearman's nonparametric rank correlation test (15) was used to examine the relationship between enzyme levels and relative susceptibility to the peach canker pathogens (see 6). Ranks were established among cultivars for the timing of appearance and maintenance of the wound response protein and these were correlated with the ranks of relative susceptibility to peach canker.

#### Results

Four peroxidase isozymes were present in all samples. The following text will refer to each of these isozymes by number, 1 to 4, one and four being the slowest and the fastest migrating enzymes respectively, with 2 and 3 having intermediate rates of migration. Representative gels from each cultivar are shown in Fig. 1.

**'Earlired'**

Peroxidase 1 levels were highest at 0 h and declined at 4 h and 1 day post-wounding. Levels of this isozyme started to increase at 48 h and then remained fairly constant through day 10. Levels of this isozyme increased appreciably by day 14. Peroxidases 2, 3, and 4 showed no quantitative changes except for an increase in activity at day 14 for isozymes 3 and 4. Enzyme responses in the second experiment were similar to those in the first experiment except that peroxidase 1 levels appeared to drop slightly at 4 and 10 days post-wounding.

**'Vanity'**

The level of activity of isozyme 1 increased at 1 and 24 days. The levels of isozyme 2 decreased slightly at 0 h and 7 days. Peroxidase isozymes 3 and 4 were depressed at 2 days, otherwise the levels of activity did not change throughout the course of the experiment. In the second experiment, the level of isozyme 1 decreased at 14 days. The activity of isozyme 2 was similar to that of isozyme 1. No changes in the levels of isozymes 3 and 4 could be observed through the course of the second experiment.

**'Redhaven'**

The level of peroxidase 1 remained constant through day 7, dropped below day 0 levels by day 10, then returned to day 0 levels by day 14. Peroxidase 2 levels were unchanged through day 7 and decreased at 10 and 14 days post-wounding. Peroxidases 3 and 4 exhibited changes similar to peroxidase 2. Enzyme responses in the second experiment were similar to those in the first experiment.

**'Vivid'**

High levels of isozyme 1 were observed at 0 through 2 days. Levels decreased at 96 h and increased at 7 and 10 days post-wounding. Data from day 14 were unavailable for this culti-

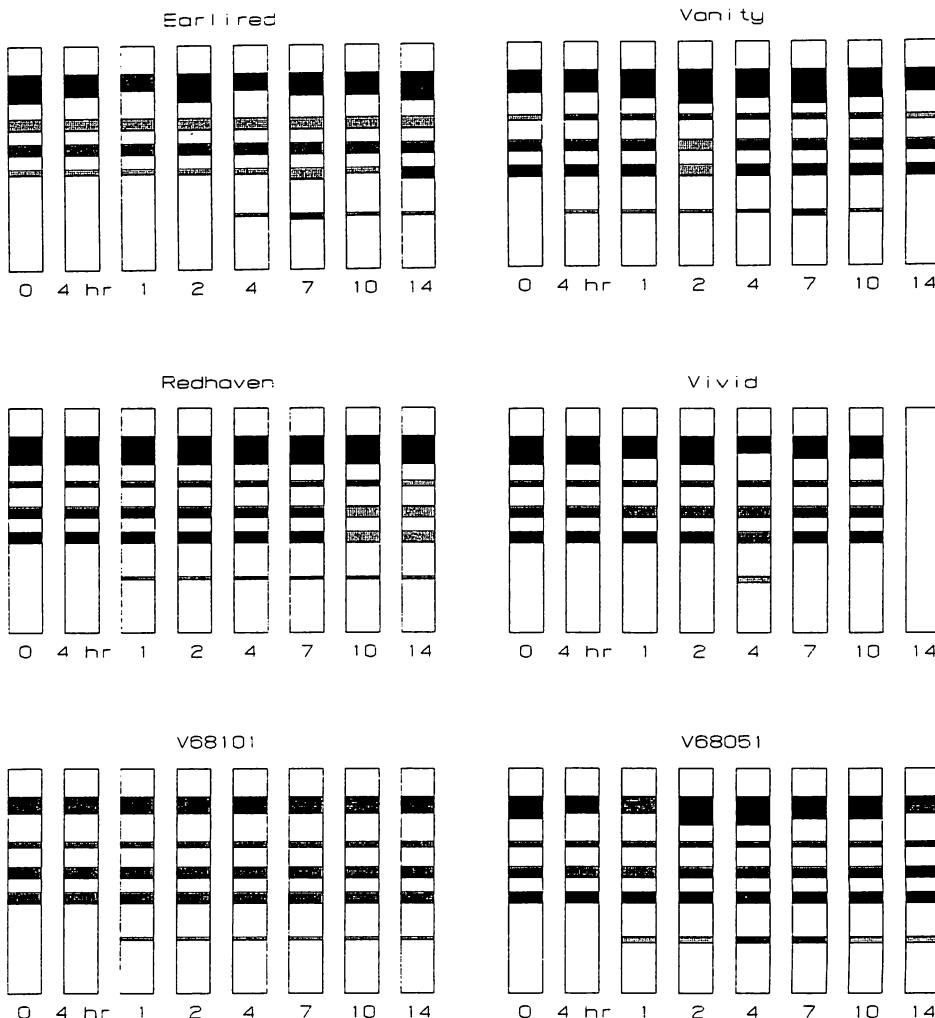
var. For isozyme 3, high levels were observed at 0 and 4 h, decreasing at 1-4 days, and increasing again at 7 and 10 days post-wounding. No changes in the quantities of isozymes 2 were observed. Isozyme 4 decreased at 4 days. In experiment 2, high levels of peroxidase 1 were observed at 0 and 4 h followed by decreases at 1 and 2 days. Isozyme levels rose again at 4 days and 7 days then decreased again at 10 and 14 days. No changes were observed for isozyme 2. High levels of isozyme 3 were observed at 0 h, decreasing at 4 h, increasing from day 1 through day 7, then decreasing again at 10 and 14 days post-wounding. Peroxidase 4 behaved in an identical manner to peroxidase 3.

**V68101**

Clone V68101 exhibited the lowest peroxidase activity of all the tested cultivars and clones. This clone also displayed the least difference in the pattern of response compared to the other cultivars. Levels of isozyme 1 appeared to stay constant throughout the course of this experiment and no changes in the activity of isozymes 2, 3, and 4 were observed throughout the course of the first experiment. In the second experiment, the level of peroxidase 1 stayed constant through the course of the experiment except for increases at 4 and 7 days of post-wounding. No changes in the levels of isozymes 2, 3, and 4 could be detected in the second experiment.

**V68051**

Levels of peroxidase 1 were highest at day 0 with quantities decreasing at 4 h and 1 day post-wounding. Isozyme levels were increased at 2, 4, and 7 days post-wounding, then declined again at 10 and 14 days. Levels of peroxidase 3 were depressed after 4 h and 1 day relative to 0 h then increased steadily from 2 to 14 days post-wounding. No changes could be detected in isozymes 2 and 4 in response to



**Figure 1.** Diagrammatic representation of polyacrylamide slab gels for the four peach cultivars and two clones examined for bark isoperoxidase activity at 0, 4 hr, and 1, 2, 4, 7, 10, and 14 days after mechanical wounding in June 1989 (Experiment 1). Bands from top to bottom of figures represent isozymes 1-4 and the wound response protein, respectively.

wounding. Isozyme responses in the second experiment generally were similar to those in the first experiment.

Total peroxidase activity is shown for each cultivar in Fig. 2. Results from the factorial analysis of variance indicated that there were no significant interactions among cultivar, time post-wounding and experiment. Mean

absorbence values for peroxidase activity were 1.46 and 1.41 for the first and second experiment, respectively. The main effects of cultivar and time were significant ( $P \leq 0.0001$ ). For cultivar (in descending order), 'Vanity,' 'Redhaven,' clone V68051, and 'Vivid' exhibited higher levels of mean peroxidase activity than 'Earlired' and clone

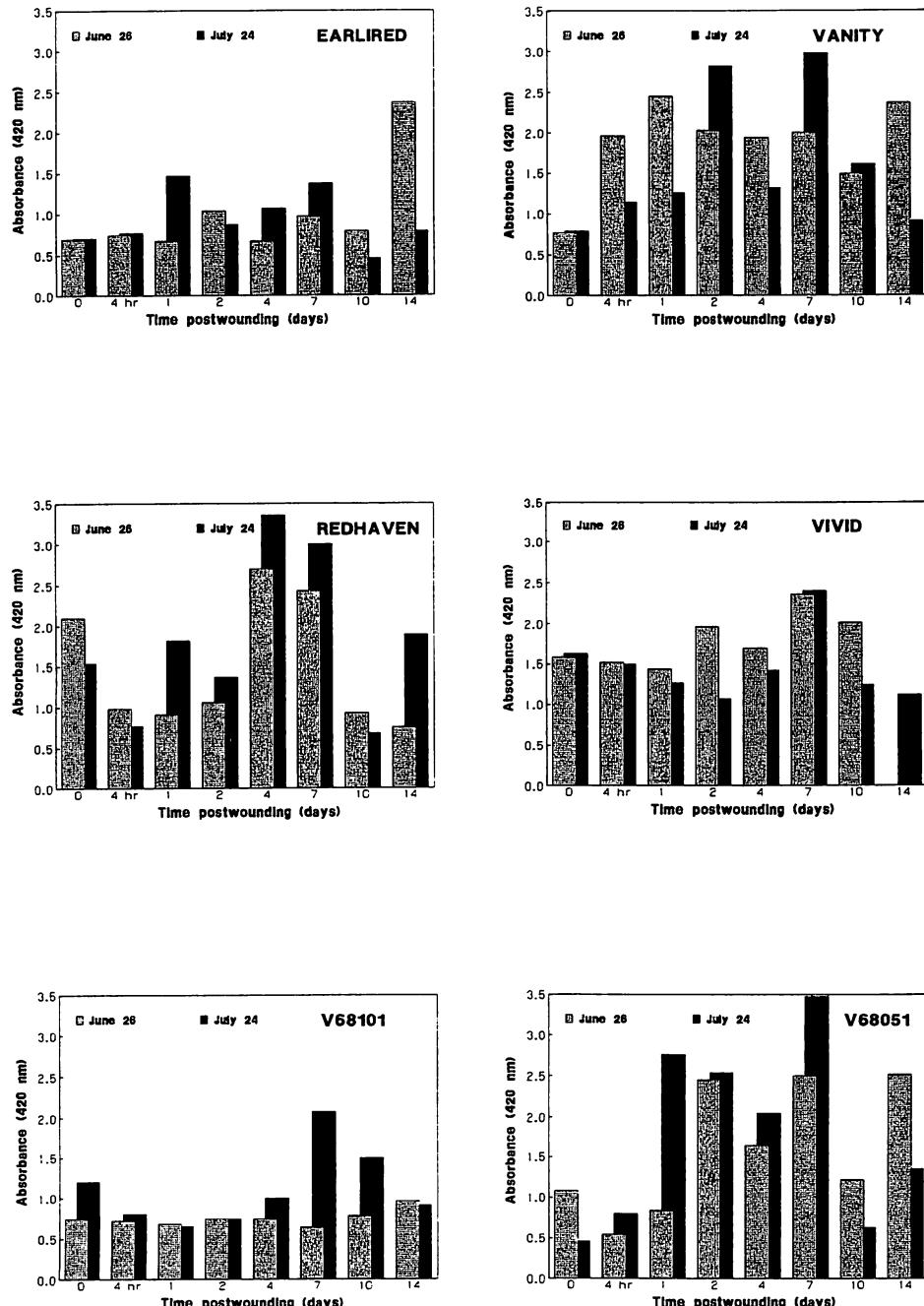


Figure 2. Spectrophotometric analysis (absorbance 420 nm) of peroxidase activity in the bark of six peach cultivars at 0, 4 hr, and 1, 2, 4, 7, 10, and 14 days after wounding in two experiments conducted in June and July 1989.

V68101, which were similar. For time post-wounding, mean peroxidase activity peaked at 7 days post-wounding, with a value significantly higher ( $P \leq 0.05$ ) than that observed at any other time. The correlation of ranks of mean peroxidase activity for cultivars with ranks for field performance viz. susceptibility to *Leucostoma* spp. was not significant (Spearman's  $r = -0.09$ ).

Changes in total peroxidase activity seems to be most affected by changes in the levels of peroxidase isozyme 1 when the electrophoretic data is compared to the spectrophotometric data. Surprisingly, the two clones, which in the field show the highest degree of resistance to the canker pathogens, have very different peroxidase profiles in responses to wounding. Clone V68051 shows the highest peroxidase activity per  $\mu\text{g}$  protein whereas clone V68101 shows the lowest peroxidase activity per  $\mu\text{g}$  protein. The two clones are both derived from a seedling of the cross NJC1 X Hui Hun Tao (hereafter referred to as NJC1HHT). V68101 resulted from open-pollination of NJC1HHT, and V68051 is a seedling of Babygold7 X NJC1HHT.

An unidentified wound response protein (WRP) was detected during the course of the isozyme experiments (Fig. 1). The WRP showed no reaction in gels stained for peroxidase activity but was detected in gels stained with Coomassie blue. In 'Redhaven,' the WRP first appeared at 24 h and levels rose to their highest at 7 days then dropped after 10 days. The appearance of the WRP in the second experiment was identical that in the first experiment. For 'Earlired,' the WRP first appeared at 96 h post-wounding and reached maximum intensity at 7 days. Levels of this protein started to decline at 10 and 14 days post-wounding. The response of WRP in the second experiment was identical to that in the first. For clone V68051, WRP first appeared at 24 h and attained its highest levels at 96 h and 7 days post-

wounding. Levels declined at 10 and 14 days although the WRP could still be detected. In the second experiment, WRP was present at a low level at 0 h but increased steadily at 24 h through 14 days post-wounding. With clone V68101, WRP was present in much lower amounts than any other clone or cultivar. In both experiments, the WRP from V68101 was observed from 24 h through 14 days with unchanging levels. Wound response protein for 'Vivid' was visible only at 96 h in experiment 1 and only at 96 h and 7 days in experiment 2. For 'Vanity,' WRP first appeared at 4 h and increased until day 10. WRP was not detected at 0 h and 14 days. In experiment 2, WRP was first observed at 48 h and achieved maximum activity at 7 days. WRP was not detected at 14 days.

### Discussion

Changes in peroxidase activity in response to mechanical wounding were observed in this study. The observation that peroxidase activity was, in most cases, highest at 7 day post-wounding is correlated generally with the appearance of ligno-suberized wound periderm (4). The fact that some cultivars showed a decrease in peroxidase activity shortly after wounding may be due to the activity of proteases released from ruptured cells at the time of wounding (12). Released proteases would degrade proteins in the perimeter of the wound, thus rendering local proteins nonfunctional without changing the total protein content (as determined by the Biorad assay) of the bark. Therefore, equivalent amounts of protein determined for the protein extracts would differ in the amount of active enzyme. These proteases are probably active for a short period, i.e. 4 to 24 h, and the subsequent increases in peroxidase activity is probably due to de novo synthesis of enzyme by cells surrounding the wound which are sufficiently protected from the proteases but which

are responding to the injury of nearby cells. Higher protease activity may explain why less peroxidase activity was observed in V68101.

The differences in the peroxidase activity profiles of the two most resistant cultivars (V68101 and V68051) suggests two types of mechanisms for responding to injury, namely an inducible and constitutive mechanism. There is precedent for the existence of both systems (16). An inducible system would involve the synthesis and maintenance of increased amounts of peroxidase for a period of time sufficient to heal the wound. An alternate system would involve a process whereby peroxidases are produced constitutively at low levels. In this way, the plant is always ready to respond immediately to a wound and no lag time between injury and response would be necessary.

The existence of multiple injury response mechanisms is suggested by the production of the wound response protein. The timing of the appearance and maintenance of this protein is correlated with resistance to the canker pathogens (Spearman's  $r = 0.95$ ,  $P = 0.01$ ). The wound response protein is produced in each cultivar in the following order: V68101 and V68051 (early production at 24 h and maintenance throughout the sample period), 'Vanity' and 'Redhaven' (early to intermediate times of production followed by rapid decline at 7 to 10 days post-wounding), 'Vivid' and 'Earlired' (late production and rapid decline). This ranking matches field tests which place V68101 and V68051 as the two least susceptible clones, 'Vanity' and 'Redhaven' are of intermediate susceptibility, and 'Vivid' and 'Earlired' are the most susceptible (5, 6). Whether the response mechanism(s) in peach involve the production of proteinase inhibitors (7, 9, 14), stress response proteins (11), or some other product remains to be determined.

The activity of peroxidase in response to wounding seems to be reproducible. However, comparisons of the types of response of one cultivar to another provided no indication that peroxidase profiles could be used as a marker for resistance to pathogens during the summer. The highest mean peroxidase activity values observed at 7 days post-wounding may be related to wound periderm formation, which has been shown in other studies to be very active at during the 4 to 10 day period after wounding (4, 6). In the months of May and June in Ontario, it was shown that cultivar relative susceptibility to *Leucostoma* spp. was significantly correlated with suberin accumulation rate in the wound periderm, and that this relationship ceased to be significant during the summer months (6). Future experiments to examine potential resistance markers should be conducted during this period. In addition, more in-depth studies on the nature of the wound response protein are required to determine its potential as a marker for pathogen resistance.

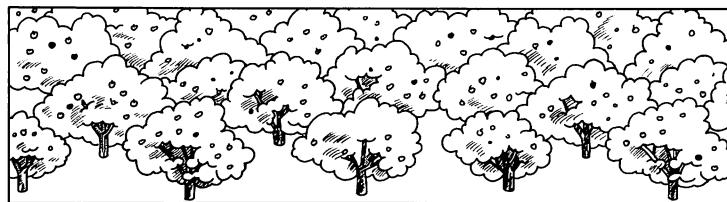
#### Acknowledgement

This research was conducted while the second author was Research Scientist and Head, Plant Pathology Section, Agriculture Canada, Research Station, Vineland Station, Ontario, Canada, L0R 2E0. The authors are grateful for the assistance and advice of Ken Slingerland, Rob Boulienne, and Drs. Neil Miles and Antonet Svircev.

#### Literature Cited

1. Biggs, A. R. 1984. Boundary zone formation in peach bark in response to wounds and *Cytospora leucostoma* infection. *Can. J. Bot.* 62:2814-2821.
2. Biggs, A. R. 1986a. Prediction of lignin and suberin deposition in boundary zone tissue of wounded tree bark using accumulated degree days. *J. Amer. Soc. Hort. Sci.* 111:757-760.
3. Biggs, A. R., 1986b. Comparative anatomy and host response of two peach cultivars inoculated with *Leucostoma cincta* and *L. persoonii*. *Phytopathol.* 76:901-912.

4. Biggs, A. R. 1986c. Wound age and infection of peach bark by *Cytospora leucostoma*. Can. J. Bot. 64:2319-2321.
5. Biggs, A. R. 1989. Temporal changes in the infection court following wounding of peach bark are associated with cultivar variation in infection by *Leucostoma personii*. Phytopathology 79:627-630.
6. Biggs, A. R., and Miles, N. W. 1988. Association of suberin formation in uninoculated wounds with susceptibility to *Leucostoma cincta* and *L. personii* in various peach cultivars. Phytopathology 78:1070-1074.
7. Bowles, D., 1990. Signals in the wounded plant. Nature 343:314-315.
8. Flott, B. E., Moerschbacher, B. M., and Reisener, M. J. 1989. Peroxidase isozyme patterns of resistant and susceptible wheat leaves following stem rust infection. New Phytol. 111:413-421.
9. Graham, J. S., Hall, G., and Ryan, C. A. 1986. Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta 169:399-405.
10. Kumar, S., and Goswami, A. K. 1985. Changes in peroxidase-IAA oxidase in developing peach fruits. Ind. J. Plant Physiol. 28:331-336.
11. Lindquist, S. and E. A. Craig. 1988. The heat shock proteins. Ann. Rev. Genet. 22:631-677.
12. Pearce, G., Ryan, C. A., and Liljegren, D. 1988. Proteinase inhibitors I and II in fruit of wild tomato species: Transient components of a mechanism for defense and seed dispersal. Planta 175:527-531.
13. Racusen, D. and M. Foote. 1965. Protein synthesis in dark-grown bean leaves. Can. J. Bot. 43:817-824.
14. Shimoni, M., and Reuveni, R. 1988. A method for staining and stabilizing peroxidase activity in polyacrylamide gel electrophoresis. Anal. Biochem. 175:35-38.
15. Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. 2nd ed. McGraw-Hill, New York.
16. VanHuyse, R. B., 1987. Some molecular aspects of plant peroxidase biosynthesis studies. Ann. Rev. Plant Physiol. 38:205-219.



Take away the commitment to quality,  
the tradition of honesty, and the history  
of superior service, and there's still thousands  
of reasons to come to ACN.

Write or call for free catalog • Adams County Nursery • P.O. Box 108  
Nursery Road • Aspers, PA 17304  
(717) 677-8105 • (717) 677-4124 FAX

