Influences of Cross- and Self-pollination on Peroxidase Activities, Isozymes, and Histological Localization during ‘Sharpblue’ Blueberry Fruit Development

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ADDITIONAL INDEX WORDS. *Vaccinium corymbosum*, isoperoxidase, tissue printing

ABSTRACT. Five-year old ‘Sharpblue’ southern highbush blueberry plants (*Vaccinium corymbosum* L.) were self- and cross-pollinated (‘O’Neal’) to study peroxidase (POD) activity, isozyme patterns, and histological localization during fruit development. Cross-pollination resulted in larger and earlier-ripening fruit. Activities of soluble and bound POD were very high during fruit growth period I, with peaks at 10 and 20 days after self- and cross-pollination. Activity was much higher for cross-pollinated fruit. During fruit growth period II, POD activities were low in both pollination treatments. During ripening, soluble POD increased, then declined in both treatments. Bound POD activities increased during the color transition from blue to dark blue, with the increase greater in self-pollinated fruit. Banding patterns of soluble and bound POD isozymes and their histological localization varied by pollination treatment as well as fruit developmental stage. During fruit ripening, soluble POD activity appeared to be associated with color transition from light blue to blue, while bound POD activity appeared to be associated with color transition from blue to dark blue.

Peroxidases (PODs) have been found widely in higher plants (Campa, 1991). They are among the most studied plant enzymes because of their participation in many oxidative and reductive reactions associated with physiological processes (Robinson, 1991). PODs are involved in the regulation of fruit growth and development via metabolic roles in lignin biosynthesis (Goldberg et al., 1991), indoleacetic acid oxidation (Galston et al., 1968; Grambow, 1991), ripening (Seymour et al., 1993), anthocyanin catabolism, ethylene biosynthesis, chlorophyll degradation (Gaspar et al., 1991), and protection against fungal and bacterial attack (Biles and Martyn, 1993). Changes in POD patterns have also been linked with cellular differentiation (Siegel, 1993).

Genetic and physiological factors can bring about spatial and temporal changes in activities and isozyme patterns of PODs. The blueberry fruit is a good model for studying growth-related changes in POD activity and isozymes because the blueberry is a multiple-seeded fruit whose growth and development depends intimately on ovule growth and development. The ovule–berry interaction may involve POD isozymes. The ovules apparently play a critical role in berry growth and viability during the early stages after pollination. Southern highbush blueberry (SHB) cultivars have a diverse genetic background because they are interspecific hybrids of native southern species with northern highbush blueberries (*Vaccinium corymbosum*) (Lang, 1993). Therefore, they are ideal for pollination and isozyme studies. The purpose of this investigation was to describe the effect of pollen genotypes on POD activities, isozyme patterns, and histological localization during ‘Sharpblue’ blueberry fruit development.

Materials and Methods

PLANT MATERIAL AND POLLEN COLLECTION. Two SHB cultivars—Sharpblue and O’Neal—were used as pollen sources. ‘Sharpblue’ was designated as the fruiting cultivar. Five-year old plants were transferred to 37-L pots and placed in a cold-room (7.2 ± 1.1 °C) in early January 1993 to ensure sufficient chilling and synchronize blooming periods. On 15 Mar., plants were placed in a greenhouse to force flowering. Flowers were collected daily after onset of flowering. Corollas were removed, and anthers and filaments were collected with forceps. Anthers were placed in a 30 °C oven for 2 h to enhance pollen dehiscence. Debris and pollen grains were separated through a 200- μ m screen. Pollen grains were stored in sealed vials at 4 °C for 4 weeks.

POLLINATION TREATMENTS. Eight ‘Sharpblue’ plants were moved out of the cold room into a bee-proof greenhouse on 7 Apr. Flowering was monitored daily. Controlled pollination began on 21 Apr. and ended on 29 Apr. For each flower cluster, only the flowers of the same physiological age were pollinated. The remaining unopened flowers were removed. Before pollen dehiscence of the flowers that were to be pollinated, corolla and anthers were removed using forceps. Pollen was transferred onto the stigmatic surface using a camel-hair brush. Each flower was pollinated twice, early in the morning and late in the afternoon.

SAMPLE PREPARATION FOR POD ASSAYS. Berries used for POD assay and isozyme separation were picked at various days after pollination (DAP) as described in Table 1. Three individual plants served as three replications. Three sets of samples from each replication were collected at each DAP to cope with any unforeseen circumstances during the experiment. Berries were placed in an insulated container with ice and transported immediately to the laboratory. Fresh fruit mass was measured before samples were stored in a deep freezer. The sample sizes of three replications were as follows: 2 g of berries at period I (range 6 to 20 berries), two berries at period II, and one berry at period III (Table 1). Berry color descriptions are listed in Table 2 (Ballinger and Kushman, 1970; Miesle, Proctor and Lagrimini, 1991).

POD EXTRACTION. The following procedures were performed at 4

Received for publication 5 Apr. 1996. Accepted for publication 5 June 1996. Louisiana Agricultural Experiment Station manuscript no. 96-28-0095. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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Table 1. Berry sampling dates for peroxidase assay and isozyme separations during growth (I, II) and maturation (III) periods.

Pollination treatment ^a	Sampling time ^b										
	Days after pollination (DAP)										
	10	20	30	35	40	45	50	55	60	65	
SB × SB	x	x	x	x	x		x	x	x	x	
Period		I				II				III	
SB × ON	x	x	x	x	x	x	x	x			
Period		I		II		III					

^aSB = Sharpblue; ON = O'Neal.^bAn "x" means that samples were taken. Heavy labelled lines were given in the table to 'visualize' the differences in fruit growth and maturation periods of pollen genotypes.

°C or in an ice bath unless otherwise indicated. Samples were ground into fine powder in liquid nitrogen using a prechilled mortar and pestle. The powder was transferred into a centrifuge tube that contained a given amount of 50 mM sodium phosphate buffer (1:5, w/v) with 2 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% insoluble polyvinylpyrrolidone (PVP) at pH 6.0, and homogenized for 90 s. The slurry was incubated 10 min at room temperature (25 °C) and centrifuged 10 min at 11,000 g_n . The resultant supernatant fluid was retained as soluble POD fraction. The pellet was homogenized 90 s in 50 mM sodium phosphate buffer (1:5 w/v) containing 2 mM DTT, 2 mM EDTA, 0.5 mM PMSF, 1% insoluble PVP, and 0.2 M KCl at pH 6.0, and it was then incubated 10 min at room temperature (25 °C). The homogenate was centrifuged 10 min at 13,000 g_n . The supernatant was saved as ionically bound POD fraction.

ENZYME ASSAYS. Activities of ionically bound and soluble PODs were determined using guaiacol as a reducing substrate. The reaction was initiated by mixing 0.2 mL of the enzyme extract with 2.8 mL of reaction buffer, which consists of 53.7 mM guaiacol and 176 mM H₂O₂ in 50 mM sodium phosphate buffer (pH 6.0). The mixture was incubated 90 s at room temperature (25 °C). Controls were used as follows: 1) 3 mL of double deionized water (ddH₂O) to autozero; 2) Blank 1—0.2 mL of ddH₂O + 2.8 mL of reaction buffer; and 3) Blank 2—0.2 mL of enzyme fraction + 2.8 mL of ddH₂O. Enzyme activity was assayed using a spectrophotometer (lambda 4B; Perkin-Elmer). The absorbance was measured at 470 nm. For each DAP, three replications of each pollination treatment were used. Five measurements of optical density (OD) were taken for a single replication. Pooled means were used in statistical

analysis. OD values were calculated according to the following formula:

$$\{(\text{OD}_{\text{enz}} - \text{OD}_{\text{bu}} - \text{OD}_{\text{sam}}) \times (\text{Vol}_{\text{ef}}(\text{mL}) \div 0.2 \text{ mL})\} \div \text{FM} (\text{g})$$

where OD_{enz} is optical density of 0.2 mL enzyme fraction in 2.8 mL phosphate buffer; OD_{bu} is optical density of 0.2 mL ddH₂O in 2.8 mL phosphate buffer; OD_{sam} is optical density of 0.2 mL enzyme fraction in 2.8 mL ddH₂O; Vol_{ef} is total volume of enzyme fraction (mL); and FM is fruit mass of samples (g). POD activity is expressed as unit/g FM, using $\epsilon_{470 \text{ nm}} = 26600 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for tetraguaiacol (George, 1953). One unit is defined as 100 nmol·min⁻¹.

CONCENTRATING THE ENZYME EXTRACTS. The prepared ionically bound and soluble fractions were brought to 80% ammonium sulfate saturation in an ice bath and centrifuged at 12,500 g_n for 20 min (Cooper, 1977). The supernatant was discarded. The pellet was resuspended in ≈5 mL of ddH₂O. The resuspended fraction was dialyzed 48 h against 50 mM sodium phosphate buffer (pH 5.1). After dialysis, the suspension was clarified at 8000 g_n for 10 min. The supernatant was saved and concentrated using a concentrator (Amicon Corp., Beverley, Mass.), with a molecular weight limit of 10,000 in a table centrifuge (IEC HN-SII) at 0 °C until the final volume of ≈300 µL was reached.

ISOELECTRIC FOCUSING. Electrode solutions were prepared as described by Righetti (1983). Acrylamide gel was prepared as described by Whitmore (1990), containing 2% ampholyte with the pH range from 3 to 10 (Pharmalyte, Sigma). Power for isozyme separation was furnished by a 1000-V microprocessor power supply (Haake Buchler Instruments, Saddle Brook, N.J.). Flat-bed isoelectric focusing was carried out with a submarine gel system (model EC360; E-C Apparatus Corp., St. Petersburg, Fla.) as

Table 2. Descriptions of berry color changes during sampling.

Treatment ^a	Days after pollination	Color descriptions
SB × ON	35	Percentage of berry surface red at the calyx end = 40%–50%
	40	The whole berry surface turning red
	45	Light blue but partially red at the calyx end
	50	Totally blue on the whole berry surface
	55	Dark blue and fully ripe
SB × SB	40	Green
	50	Completely red on the whole berry surface
	55	Light blue
	60	Totally blue on the berry surface
	65	Dark blue and fully ripe

^aSB = Sharpblue; ON = O'Neal.

described by Whitmore (1990) and Lagrimini and Rothstein (1987), with some modifications. The gel was prefocused 30 min at 200 V, then loaded with protein samples equivalent to 100 mg FM in each lane and focused 5 h at 0 °C with constant wattage (4 W for 1 h, and 5 W for 4 h). POD isozymes were stained as described by Miesle et al. (1991). The gel was washed 30 min with phosphate buffer saline (1× PBS), which consisted of 10 mM of sodium phosphate buffer (pH 6.0) and 150 mM of NaCl, to remove the ampholytes and achieve uniform pH throughout the gel. Isozyme bands were made visible by incubating at 30 °C in 1× PBS containing 0.6 mg 4-chloro-1-naphthol per mL and 0.18% H₂O₂. Blue bands were observed after about 20 min incubation.

HISTOLOGICAL LOCALIZATION OF POD ACTIVITY. POD activity distribution in self- and cross-pollinated 'Sharpblue' blueberries was studied by tissue printing on nitrocellulose paper. The purpose of doing this was to look for additional evidence to support the original hypothesis that blueberry fruit growth and development was associated with POD activity, which was affected by pollen genotype. A tissue-printing protocol was developed to study the profiles of histological localization of POD activity at fruit growth period I and at ripening. Phosphate buffer and chemicals used for detection of POD activities were the same as used for detecting isozyme bands on acrylamide gels. Triple controls were used: 1) 50 mM phosphate buffer only (pH 6.0); 2) 50 mM phosphate buffer with 0.18% hydrogen peroxide (H₂O₂) added; 3) 50 mM phosphate buffer with 0.6 mg 4-chloro-1-naphthol/mL added. The solution for detection of POD activity was 50 mM phosphate buffer containing 0.6 mg 4-chloro-1-naphthol/mL and 0.18% H₂O₂. The method was as described by Varner (1992) with some modifications. 1) A piece of 5×3-cm nitrocellulose paper was soaked 10 min in 0.2 M CaCl₂ solution and placed on 3-mm Whatman no. 4 paper to dry. 2) The berry was cut longitudinally in half, washed in distilled water for a few seconds, and dried on tissue. 3) The section was placed on nitrocellulose paper using forceps and pressed for 60 s using a gloved fingertip. The nitrocellulose paper with the tissue printing was dried immediately by warm air flow. 4) It was then placed in 50 mM phosphate buffer containing 0.6 mg 4-chloro-1-naphthol/mL and 0.18% H₂O₂. A blue color became visible after ≈25 min incubation. This blue insoluble product on nitrocellulose paper indicated the location of POD activity in the berry. No blue color was detected in the triple controls after 25 min incubation, indicating that the blue color observed above was not caused by nonenzymatic reactions. 5) The nitrocellulose paper was dried and photographed.

DATA PROCESSING. Data of fruit mass and POD activities derived from self- and cross-pollinations were analyzed by general linear model procedure (SAS Institute, Cary, N.C.). Mean separations were accomplished by Duncan's multiple range test. Data of fruit mass and POD activities at fruit growth period II were subject to linear regression analysis.

Results and Discussion

FRUIT GROWTH AND POD ACTIVITY. Growth curves and POD activity changes of self- and cross-pollinated 'Sharpblue' blueberries demonstrated three distinct periods (Figs. 1 and 2). The fruit growth curve was described as follows: period I, a time of slow growth, concomitant with ovary growth and development; period II, an exponential increase in fruit mass and endocarp lignification; and period III, slowing fruit growth followed by ripening. These three periods were altered by pollen genotype. Self-pollinated berries required 40 d to complete period I, whereas it took only 30 d for cross-pollinated berries. Cross-pollinated berries had a shorter period II with a greater fruit growth rate than self-pollinated

Fig. 1. Comparison between changes in soluble peroxidase activity and fruit mass (FM) of self- and cross-pollinated 'Sharpblue' blueberries. Self = self-pollinated 'Sharpblue' fruit; period I = 10 to 35 days after pollination (DAP); period II = 35 to 50 DAP, and period III = ripening. Cross = cross-pollinated 'Sharpblue' fruit with 'O'Neal' as a pollen source; period I = 10 to 30 DAP; period II = 30 to 40 DAP, and period III = ripening.

Fig. 2. Comparison between changes in bound peroxidase activity and fruit mass (FM) of self- and cross-pollinated 'Sharpblue' blueberries. Self = self-pollinated 'Sharpblue' fruit; period I = 10 to 35 days after pollination (DAP); period II = 35 to 50 DAP, and period III = ripening. Cross = cross-pollinated 'Sharpblue' fruit with 'O'Neal' as a pollen source; period I = 10 to 30 DAP; period II = 30 to 40 DAP, and period III = ripening.

berries. For cross-pollination, average berry mass increased from 0.48 to 1.78 g (272%) within 10 d. It took 15 d for self-pollinated berries to increase fruit mass from 0.5 to 1.35 g (167%). Also, cross-pollination resulted in larger and earlier-ripening fruit. Cross-pollinated berries were 51% larger at maturity and ripened about 10 d earlier than self-pollinated berries. Similar results were reported by Gupton and Spiers (1994). Their study indicates that self-pollination of 'Sharpblue' and other SHB cultivars significantly decreases berry mass and increases fruit development period (FDP); whereas, using 'O'Neal' and 'Gulfcoast' as pollen parents on other SHB cultivars results in larger berries and shorter FDP than with self-pollination.

This study also demonstrated that pollen genotypes could affect POD activity throughout fruit development. On a fruit mass basis,

activities of soluble and bound PODs were very high during early fruit development, with peaks at 10 and 20 d after self- and cross-pollination, respectively. Peak activity in cross-pollination was higher. Coincidentally, cross-pollination showed a shorter growth period I and significantly greater fruit mass during early fruit development. During period II, fruit growth differed by ≈ 5 d between pollination treatments; POD activity from self- and cross-pollination was generally low (Figs. 1 and 2).

Soluble POD activity positively and negatively correlated with fruit mass in self- and cross-pollination, respectively; and no correlations existed between bound POD activity and fruit mass in either treatment (Fig. 3).

Changes in soluble and bound POD activities were also different between pollination treatments (Table 3). Bound POD activity did not show obvious changes in either pollination treatment, whereas soluble POD activity significantly increased in self-pollinated and decreased in cross-pollinated fruit at the end of period II. There was a greater fruit growth corresponding to the decline in soluble POD activity in cross-pollination during this period. During fruit ripening (period III), soluble POD activity reached the second peak at berry color transition stage (40 DAP) in cross-pollination (Fig. 1); activity significantly declined at 45 DAP, followed by a slight rise till a significant increase at full ripening. Soluble POD activity in self-pollinated fruit started to increase at 50 DAP, displayed a significant increase at 60 DAP, then a slight decrease at 65 DAP. During this period, fruit color changed from red to dark blue. Bound POD activity significantly increased during color transition from blue to dark blue for both pollination treatments, with the increase being much greater in self-pollinated berries.

ELECTROPHORETIC ANALYSIS OF ISOZYMES. Several isozymes with different staining intensities were detected in both pollination treatments during fruit development (Figs. 4 and 5). Six and five soluble POD bands (S1, S2, S3, S4, S5, S6, and S3') were detected for cross and self-pollination, respectively (Fig. 4). The isoforms S4 and S5 appeared at 20 and 30 DAP in cross-pollinated berries only, and S3', which was unique to self-pollinated fruit, was detected at periods I and III. The isoform S6 was detected at 20 DAP in cross-pollinated fruit and at 10 DAP in self-pollinated fruit. The soluble isozymes S1, S2, and S3 disappeared at certain developmental stages: S1 at 45 and 50 DAP in cross-pollinated fruit and at 35 through 55 DAP in self-pollinated fruit; S2 at 10 DAP; and S3 at 10 and 55 DAP in cross-pollinated fruit. Four bound POD bands (B1, B2, B3, and B4) were observed for both pollination treatments (Fig. 5). Some bound isozymes disappeared at certain developmental stages: B1 at 10 DAP, B3 at 20 and 45 DAP, and B4 at 20, 30, 45, and 50 DAP in cross-pollinated fruit and B1 at 20 DAP, B2 at 20 through 35 DAP, and B4 at 30, 40, and 50 DAP in self-pollinated fruit.

HISTOCHEMICAL LOCALIZATION OF POD ACTIVITY. POD activity and histological location were reflected by the color intensity revealed in the individual photographs (Figs. 6–8). POD activity was hardly

detected in unopened flowers [Fig. 6(1) and 6(2)], and was present primarily at the calyx end at 3 DAP for self- and cross-pollinated berries, with a higher activity in self-pollinated fruit, as implied by the color intensity in Fig. 6(4). This is sound evidence that pollination can stimulate expression of enzymes needed for fruit growth and development. The activity in cross-pollinated berries was higher than in self-pollinated berries at 5 and 10 DAP. At 10 DAP [Fig. 6 (10 to 12)], the activity was detected in the peripheral region of the berry. The peripheral tissue of the fruit revealed most of the POD activity at 20 and 30 DAP for both pollination

treatments, with cross-pollinated fruit showing higher activity (Fig. 7). In the previous experiments, isoelectric focusing showed more and different isozymes (Fig. 4), and activity assays indicated the presence of higher total, as well as bound, POD activities at 20 and 30 DAP for cross-pollination (Figs. 1 and 2). Coincidentally, cross-pollination resulted in greater fruit mass at 20 and 30 DAP.

Although the exact functions of PODs in plants are elusive, all these facts indicate that PODs participate in fruit growth and development and that the expression pattern of these isozymes is tissue specific and developmentally regulated.

As shown in Fig. 8, tissue prints revealed the localization of POD activity in the berry and the different distribution pattern between self- and cross-pollination treatments during the color transition from red to dark blue. When berries turned red, POD activity was located mainly in the peripheral region and calyx end and slightly along the calyx-stem axis in self-pollinated berries. In cross-pollinated fruit, the POD activity increased and was distributed from the stem end to the calyx end (Fig. 8). At the blue stage,

Fig. 3. Relationship between 'Sharpblue' blueberry fruit mass (FM) and peroxidase (POD) activities derived from self- and cross-pollination ('O'Neal') during fruit growth period II. (A) FM vs. soluble POD activity. $FM_{(self)} = 0.368 + 0.471POD$ ($R^2 = 0.98$, slope is significantly different from zero at $P < 0.001$). $FM_{(cross)} = 2.630 - 0.221POD$ ($R^2 = 0.95$, slope is significantly different from zero at $P < 0.01$). (B) FM vs. bound POD activity. $FM_{(self)} = 2.965 - 0.129POD$ ($R^2 = 0.15$, no regression relation). $FM_{(cross)} = 6.285 - 0.353POD$ ($R^2 = 0.55$, a weak regression relation).

self-pollinated fruit showed POD activity mostly at the stem end and along the calyx-stem axis, with a slight increase in activity, while cross-pollinated fruit displayed the activity throughout the flesh tissue. In fully ripened fruit (dark blue), very high activity was detected throughout self- and cross-pollinated fruit. The results from tissue printing agreed with the results of the activity assays, when expressed as total activity per berry. The results from this study indicated that POD activity increased and its histological location changed with fruit ripening.

A large body of literature has reported the versatility of PODs in physiological roles. Different isoperoxidases may be involved in separate physiological processes during fruit growth and development. More than 30 PODs could be found in a single plant variety, depending on developmental stages and tissues (Siegel, 1993). More than 40 isoperoxidases were resolved in callus and vegetative and floral buds of *Nicotiana tabacum*, and half of the isozymes were correlated with specific developmental events (Kay and Basile, 1987). Only 11 POD isozymes were detected in this study. It was possible for some isozyme forms to have escaped from detection in this study, since only a single substrate was used for detection of the isozymes. Kay and Basile (1987) used different substrates to detect POD isozymes in *Nicotiana tabacum* and found that about 43% of the bands escaped from detection when using guaiacol instead of *o*-dianisidine.

The results of activity assays and tissue printing indicated that cross-pollination resulted in much higher activities for both classes of PODs, and that the fruit growth curve showed a greater fruit mass during fruit growth period I. The rapid increase of northern highbush fruit volume at stage I is attributed to cell division and some enlargement (Gough, 1994). Therefore, we postulate that a cross-pollinated berry probably underwent cell division more rapidly than a self-pollinated berry. The rapid cell division as well as enlargement would contribute to the differences in fruit mass and require higher POD activity for

Table 3. Peroxidase (POD) activities and fruit mass affected by pollination treatments of 'Sharpblue' fruit at the onset and end of growth period II.

Fruit developmental period	Self-pollination POD activities			Fruit mass (g)	Cross-pollination POD activities			Fruit mass (g)
	Soluble (units)	Bound (units)	Total		Soluble (units)	Bound (units)	Total	
Onset	0.3 b ²	16.3 a	16.6 a	0.50 b	9.5 a	15.5 a	25.0 a	0.49 b
End	2.1 a	15.5 a	17.6 a	1.35 a	4.0 b	13.6 a	17.6 b	1.78 a
Significance	*	NS	NS	**	*	NS	*	**

²Mean separation within columns at $P < 0.05$ by Duncan's multiple range test.

NS,*,** Nonsignificant or significant at $P < 0.05$ or 0.01 , respectively.

cell-wall biosynthesis. There is evidence to support this hypothesis. Recent studies indicate that biosynthesis of lignin (a major chemical component of the cell wall) requires the participation of

bound and soluble PODs (Campa, 1991). Gough (1983) reported a concentration of stone cells in the periphery of cultivated high-bush berries that apparently differentiate from ground parenchyma of the mesocarp shortly after anthesis. Stone cell walls contain large amounts of lignin. PODs are abundant in cell walls. Soluble isoperoxidases have been reported to contribute to the cell-wall POD activity, since they show high affinity for lignin precursors. Isozymes of bound PODs are found only in vacuoles. They show low activity for lignin precursors, but are capable of catalyzing the formation of hydrogen peroxide. This suggests that the two isozyme fractions work cooperatively in cell-wall biosynthesis. Bound PODs produce hydrogen peroxide, which is used by soluble PODs to generate free-radical intermediates from lignin precursors. The physiological roles of PODs in cell-wall metabolism are far more extensive than in lignin biosynthesis. They participate in cell-wall metabolism by 1) cross-linking tyrosine residues of extensin (an abundant cell-wall protein) and possibly other wall glycoproteins, 2) cross-linking hydroxycinnamic acid and *p*-hydroxybenzoic acid residues of pectins and certain xylans, 3) linking extensin to polysaccharides, 4) catalyzing lignin binding to cell-wall glycoproteins, and 5) suberization (Gaspar et al., 1991).

1-Aminocyclopropane-1-carboxylic acid oxidase is a key enzyme at the last step of ethylene biosynthesis. This enzyme is postulated to be a POD (Gaspar et al., 1991). Many aspects of plant growth and development can be regulated by ethylene. One of its major roles is to initiate fruit ripening. Ethylene is involved in biosynthesis of many ripening-associated enzymes. Therefore, the increase in POD activity during berry ripening was probably associated with ethylene biosynthesis. POD involvement in ethylene production in plants is based on the following experiments. 1) Horseradish (*Armoracia rusticana*) POD

Fig. 4. Isozyme bands of soluble peroxidases as seen in polyacrylamide electrophoresis gels. For color transition, LG = light green; R = red; LB = light blue; B = blue; DB = dark blue. Intensity of staining shown in order, from most intense to faint: dark bars, light bars, small bars.

Fig. 5. Isozyme bands of bound peroxidases as seen in polyacrylamide electrophoresis gels. For color transition, LG - light green; R - red; LB - light blue; B - blue; DB - dark blue. Intensity of staining shown in order, from most intense to faint: dark bars, light bars, small bars.

was stimulated by ethylene and inhibited by GA₃ (Trebitsh et al., 1993). POD is proposed to participate in chlorophyll catabolism, based on the fact that an increase in POD activity is observed in senescing leaves of several plants coincident with a decline in chlorophyll content (Kar and Chondhuri, 1987; Kar and Feiranbend, 1984; Birecka et al., 1979). For example, some soluble isozymes become prominent during aging, concomitant with a decline in chlorophyll content (Kar and Chondhuri, 1987.). A phenol-specific POD, bound to thylakoid, was postulated to mediate chlorophyll catabolism (Huff, 1982). These findings imply that chlorophyllase is a POD-like enzyme.

This study highlights the influences of self- and cross-pollination on fruit development and POD expression of 'Sharpblue' blueberry. The evidence indicates that pollen from different SHB genotypes can affect fruit POD activities, histological localization, and isozyme patterns during fruit growth and development.

Literature Cited

- Ballinger, W.E. and L.J. Kushman. 1970. Relationship of stage of ripeness to composition and keeping quality of highbush blueberries. *J. Amer. Soc. Hort. Sci.* 95:239-242.
- Biles, C.L. and R.D. Martyn. 1993. Peroxidase, polyphenol oxidase, and shikimate dehydrogenase isozymes in relation to tissue type, maturity and pathogen induction of watermelon seedlings. *Plant Physiol. Biochem.* 31:499-506.
- Birecka, H., M.J. Chaskes, and J. Goldstein. 1979. Peroxidase and senescence. *J. Expt. Bot.* 30:565.
- Campa, A. 1991. Biological roles of plant peroxidases: known and potential functions, p. 25-50. In: J. Everse, K.E. Everse, and M.B. Grisham (eds.). *Peroxidases in chemistry and biology*. vol. 2. CRC Press, Boca Raton, Fla.
- Cooper, T.G. 1977. The tools of biochemistry, p. 172-193. Wiley, New York.
- Galston, A.W., S. Lavee, and B.Z. Siegel. 1968. Induction and repression of peroxidase isozymes by 3-indoleacetic acid, p. 455-472. In: F. Wrightman and G. Setterfield (eds.). *Biochemistry and physiology of plant growth substances*. Runge Press, Ottawa.
- Gaspar, Th., C. Penel, D. Hagege, and H. Greppin. 1991. Peroxidases in plant growth, differentiation, and development processes, p. 249-280. In: J. Lobarzewski, H. Greppin, C. Penel, and Th. Gaspar (eds.). *Biochemical, molecular, and physiological aspects of plant peroxidases*. Univ. of Geneva, Switzerland.
- George, P. 1953. Intermediate compound formation with peroxidase and strong oxidizing agents. *J. Biol. Chem.* 201:413-425.
- Goldberg, K., A. Pang, C. Rolando, C. Francesch, and A.M. Catesson. 1991. Cell wall peroxidases and lignification: Tissue and substrate specificity, p. 209-220. In: J. Lobarzewski, H. Greppin, C. Penel, and Th. Gaspar (eds.). *Biochemical, molecular and physiological aspects of plant peroxidases*. Univ. of Geneva, Switzerland.

catalyzes ethylene production in vitro in a model system containing methional (Yang, 1967). 2) Plant extracts with IAA oxidase activity catalyze ethylene synthesis from ACC (Vioque and Vioque, 1981). PODs probably also play roles in color changes and aromatic and volatile biosynthesis during fruit ripening. In vitro use of PODs to synthesize aromatic compounds is being studied by Melo et al. (1995). In this experiment, changes in POD activity were coincident with color transition. Soluble POD activity seemed to be associated with the color transition from light blue to blue, while bound POD activity appeared to be associated with the color transition from blue to dark blue.

Fruit ripening is characterized by color changes, i.e., chlorophyll degradation coincident with the biosyntheses of anthocyanins and carotenoids (Seymour et al., 1993). Chlorophyllase is an important enzyme mediating chlorophyll degradation. In plastid fractions of orange fruit peel, de novo synthesis of chlorophyllase

- Gough, R.E. 1983. The occurrence of mesocarpic stone cells in the fruit of cultivated highbush blueberry. *J. Amer. Soc. Hort. Sci.* 108:1064–1067.
- Gough, R.E. 1994. Growth and development, p. 11–65. In: R.E. Gough (ed.). *The highbush blueberry and its management*. Food Products Press, Binghamton, N.Y.
- Grambow, H.J. 1991. Pathway and mechanism of the peroxidase-catalyzed degradation of indole-3-acetic acid, p. 31–41. In: H. Greppin, C. Penel, and T. Gaspar (eds.). *Molecular and physiological aspects of plant peroxidases*. Univ. of Geneva, Switzerland.
- Gupton, C.L. and J.M. Spiers. 1994. Interspecific and intraspecific pollination effects in rabbiteye and southern highbush blueberry. *HortScience* 29:324–326.
- Huff, A. 1982. Peroxidase-catalyzed oxidation of chlorophyll by hydrogen peroxide. *Phytochemistry* 21:261.
- Kar, R.H. and M.A. Chondhuri. 1987. Possible mechanism of light-induced chlorophyll degradation in senescing leaves of *Hydrilla verticillata*. *Physiol. Plant* 70:729–734.
- Kar, M. and J. Feiranbend. 1984. Metabolism of activated oxygen in detached wheat and rye leaves and its relevance to the initiation of senescence. *Planta* 160:385.
- Kay, L.E. and D.V. Basile. 1987. Specific peroxidase isoenzymes are correlated with organogenesis. *Plant Physiol.* 84:99–105.
- Lagrimini, L.M. and S. Rothstein. 1987. Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. *Plant Physiol.* 84:438–442.

Fig. 7. Peroxidase (POD) histological localization of self- and cross-pollinated 'Sharpblue' blueberries at 20, 25 and 30 d after pollination (DAP): 1–2 = SB × SB and SB × ON at 20 DAP, activity was mostly detected around the peripheral region of the fruit; 3–4 = SB × SB and SB × ON at 25 DAP, POD was present throughout the fruit; 5–6 = SB × SB and SB × ON at 30 DAP, POD distribution was similar to that at 20 DAP. POD activity was higher and more spread in cross-pollination than in self-pollination, as indicated by the color intensities in the photos.

Fig. 6. Peroxidase (POD) histological localization of self- and cross-pollinated 'Sharpblue' blueberries. POD activity and localization were reflected by color intensity. 1–2 = Unopened 'Sharpblue' and 'O'Neal' flowers, which showed low POD activity. 3 = Unopened 'Sharpblue' flower as a check; no activity was detected. 4–6 = SB × SB, SB × ON, and SB × GC at 3 d after pollination (DAP); 7–9 = SB × SB, SB × ON, and SB × GC at 5 DAP. POD activity was present at calyx end of the fruit. 10–12 = SB × SB, SB × ON, and SB × GC at 10 DAP; POD activity was present at calyx end as well as at peripheral region of the fruit.

Fig. 8. Peroxidase (POD) histological localization of self- and cross-pollinated 'Sharpblue' blueberries at ripening: 1 = SB × SB at red; 2 = SB × ON at red; 3 = SB × SB at blue; 4 = SB × ON at blue; 5 = SB × SB at dark blue; and 6 = SB × ON at dark blue. POD activity increased in both pollination treatments at dark blue stage.

- Lang, G.A. 1993. Southern highbush blueberries: physiological and cultural factors important for optimal cropping of these complex hybrids. *Acta Hort.* 346:72-80.
- Melo, N.S., J.M.S. Cabral, and M.P. Fevereiro. 1995. Extracellular peroxidases from cell suspension cultures of *Vaccinium myrtillus*. Purification and characterization of two cationic enzymes. *Plant Sci.* 106:177-184.
- Miesle, T.J., A. Proctor, and L.M. Lagrimini. 1991. Peroxidase activity, isoenzymes, and tissue localization in developing highbush blueberry fruit. *J. Amer. Soc. Hort. Sci.* 116:827-830.
- Righetti, P.G. 1983. Isoelectric focusing: Theory, methodology and applications. Elsevier Biochemical Press, Amsterdam.
- Robinson, D. 1991. Peroxidases and catalases in foods, p. 1-47. In: D.S. Robinson and N.A.M. Eskin (eds.). *Oxidative enzymes in foods*. Elsevier Science Publishers LTD, Crown House, England.
- Seymour, G.B., J.E. Taylor, and G.A. Tucker. 1993. *Biochemistry of fruit ripening*. Chapman & Hall, London.
- Siegel, B.Z. 1993. Plant peroxidases, an organismic perspective. *Plant Growth Regulat.* 12:303-312.
- Trebitsh, T., E.E. Goldschmidt, and J. Riov. 1993. Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. *Proc. Natl. Acad. Sci.* 90:9441-9445.
- Varner, J.E. 1992. Visualization of enzyme activity, p. 59-70. In: P.D. Reid (ed.). *Tissue printing: Tools for the study of anatomy, histochemistry, and gene expression*. Academic Press, Harcourt Brace Jovanovich, San Diego.
- Vioque, A.A. and B. Vioque. 1981. Role of IAA oxidase in the formation of ethylene from 1-aminocyclopropane-1-carboxylic acid. *Phytochemistry* 20:1473-1475.
- Whitmore, D.H. 1990. Isoelectric focusing of proteins, p. 81-105. In: D.H. Whitmore (ed.). *Electrophoretic and isoelectric focusing techniques in fisheries management*. CRC Press, Boca Raton, Fla.
- Yang, S.F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. *Arch. Biochem. Biophys.* 122:481.