Changes in Polyamine Levels in Relationship to the Double-sigmoidal Growth Curve of Peaches

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ABSTRACT. Polyamines and the activities of their biosynthetic enzymes were evaluated during peach (*Prunus persica* L. 'Biscoe') mesocarp (pulp) and seed growth starting at full bloom and until full fruit maturity at 14 weeks after full bloom (AFB). Mesocarp fresh mass exhibited a double-sigmoidal pattern characteristic of peaches. Seed fresh mass increased to a maximum of ≈ 1 g at 4 weeks AFB then remained unchanged during the remaining weeks of sampling. Free putrescine, spermidine, and spermine levels were significantly higher in the flower bud, declined in the mesocarp tissue during the first 2 weeks AFB, then exhibited another increase between 2 and 6 weeks AFB. In contrast, conjugated spermidine and spermine levels were low in flower buds, then increased to their maximum level at 6 weeks AFB, then declined at full fruit development. Ornithine decarboxylase (ODC, EC 4.1.1.17) activity was high in flower buds ($89.3 \text{ nmol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ protein) and in early stages of mesocarp development then declined to its lowest level ($5.8 \text{ nmol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ protein) at full-fruit development. Arginine decarboxylase (ADC, 4.1.1.19) activity did not change during the first 6 weeks of mesocarp growth but declined later, reaching its lowest ($1.95 \text{ nmol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ protein) at 14 weeks AFB. During the first 5 weeks AFB, ODC activity was 3.0- to 4.5-fold that of ADC activity; however, at full-fruit maturity (14 weeks AFB) the activities of both enzymes were similar. The slowdown in mesocarp growth during pit hardening between 6 and 9 weeks AFB did not change polyamines concentrations or their biosynthetic enzymes. Free spermidine and spermine levels declined during seed development; however, between 7 and 9 weeks AFB an increase in putrescine was observed. Similarly, conjugated putrescine increased substantially during seed growth reaching its highest level of $680 \text{ nmol}\cdot\text{g}^{-1}$ fresh mass at week 8 then declined at the later weeks, while conjugated spermidine and spermine peaked at week 10 to 1,169 and 2,148 $\text{nmol}\cdot\text{g}^{-1}$ fresh mass. ODC and ADC activities declined between 3 and 5 weeks AFB. However, a significant increase in ADC but not ODC activity in the seed tissue was observed during pit hardening between 6 and 10 weeks AFB. Based on the rapid increase in putrescine and ADC activity in the seed tissue, it appears that pit hardening may be a stress-related phenomenon. Data also suggest that polyamine levels in the mesocarp and seed tissue are independently regulated.

The polyamines spermidine, spermine, and putrescine (a diamine) are present in all living tissues (Smith, 1990). Polyamines have been reported to be essential for growth and development of living tissues through their involvement in cell proliferation, signal transduction, and in nearly every step of DNA, RNA, and protein synthesis (Kaur-Sawhney and Applewhite, 1993; Roux, 1993; Tiburcio et al., 1993). In several plant species, polyamines have been proposed to be involved in the onset and evocation of flowering (Harkess et al., 1992; Malmberg et al., 1985; Martin-Tanguy, 1985). In citrus (*Citrus sinensis* L. Osbeck) flowers, 80% of the total polyamines were found in the reproductive organs and 20% in the nonreproductive organs (Kushad et al., 1990).

Polyamines were also suggested to regulate in vitro cell differentiation (Monatgue et al., 1978). El-Hadrami and D'Auzac (1992) found that, in *Helvea brasiliensis*, inhibition of polyamine biosynthesis with difluoromethylarginine, difluoromethylornithine (DFMO), and methylglyoxal bis(guanyldrazon) reduced the endogenous pool of polyamines and lowered in vitro somatic embryogenesis. The inhibition of embryogenesis was restored when $50 \mu\text{M}$ spermidine was added to the culture medium. The presence of polyamines and their biosynthetic enzymes ornithine decarboxylase (ODC, EC 4.1.1.17) and arginine decarboxylase (ADC, 4.1.1.19) have been also linked to growth and development of several plant organs. We reported that, in avocados (*Persea americana* L.), polyamines and their biosynthetic enzymes reached

peak levels during rapid cell division then dropped to their lowest level during cell enlargement (Kushad et al., 1988). This increase in polyamines in avocados was parallel to an increase in cellular DNA. Similarly, in 'Golden Delicious' apples (*Malus domestica* Borkh), polyamines peaked during the first week after full bloom then gradually declined until they reached their lowest levels at full-fruit maturity (Biasi et al., 1988). In tomatoes (*Lycopersicon esculentum* Mill), when polyamine biosynthesis was inhibited by DFMO, cell division and cell enlargement were also inhibited (Egea-Cortines and Mizrahi, 1993). The mechanism by which polyamines regulate growth may involve their binding to specific regulatory proteins possibly present in the cell wall (Kaur-Sawhney and Applewhite, 1993).

The growth and development of stone fruit, including peaches, are different from those of fruit previously evaluated for polyamine levels. For example, the growth pattern of stone fruit (mesocarp) is different from pome fruit in that pome fruit exhibit a sigmoidal growth pattern, while stone fruit exhibit a double-sigmoidal growth pattern as a result of a period of slow growth due to pit hardening (Romani and Jennings, 1971). Also, stone fruit will not set fruit without embryo development, while pome fruit may set and grow with only a few seeds or without seed development (Westwood, 1978). The growth of peaches is also different from avocados in that avocados continue to grow throughout development by a combination of cell division and cell enlargement (Schroeder, 1953), while cell division in peaches and all other fruit ceases 2 to 6 weeks after bloom (Romani and Jennings, 1971; Westwood, 1978).

Since polyamines have been described as growth promoters and may be required by a number of cellular processes during

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growth (Smith, 1990), the possibility exists that the slowdown in growth of peaches during pit hardening may have a significant impact on polyamines synthesis. Therefore, the objective of this study was to examine changes in polyamines and their biosynthetic enzymes in relationship to peach (*Prunus persica*) mesocarp and seed development to determine if they are affected by a reported decrease in the rate of fruit growth due to pit hardening.

Materials and Methods

PLANT MATERIAL. 'Biscoe' peaches were harvested from five 15-year-old uniform size trees grafted on Lovell rootstock and trained to an open center. Trees were growing at the Univ. of Illinois Dept. of Natural Resources and Environmental Sciences Tree Fruit Research Farm, Urbana. Pruning, fertilizing, and fruit thinning were carried out according to standard orchard practices. Each tree was randomly assigned a replication number. Sampling started at full bloom and continued at weekly intervals until commercial maturity. Full bloom occurred on 27 Apr. 1996 and fruit set occurred ≈ 10 d after full bloom (AFB). Fruit were considered commercially mature when the ground color of the skin was greenish-yellow (Shade no. 3), which occurred at 14 weeks AFB. Ten fruit per tree (replication) were harvested at each sampling date, weighed, and then analyzed for polyamines and their biosynthetic enzymes. At week 3 AFB, seeds were extracted, weighed, and analyzed for polyamines and their biosynthetic enzymes.

POLYAMINE ANALYSIS. Total polyamines in the flower, flesh (mesocarp), and seed were determined separately every week, except during the first 3 weeks of sampling where they were determined in the whole fruit due to the difficulty of separating the seed from the mesocarp. About 5 g of fully opened flowers or 5 g of fresh mesocarp tissue from the 10 fruit (0.5 g per fruit) per replication at each sampling date were homogenized separately in 25 mL of 5% HClO_4 . The seeds from each fruit per replication were extracted from the pit and homogenized in 25 mL of 5% HClO_4 . Tissues were homogenized with a Polytron homogenizer (Brinkman Inst. Co., Westbury, N.J.) set at a speed of 7. Homogenates were centrifuged at 20,000 g_n for 30 min, and the supernatant was assayed for free (water soluble) polyamines according to Flores and Galston (1982) and as modified by Kushad et al. (1990). An aliquot of 300 μL was placed in a 25-mL conical Pyrex tube and 2 mL of 2 N NaOH plus 5 μL benzoyl chloride were pipetted into each tube. The mixture was vortexed for 10 s and incubated at 30 $^\circ\text{C}$ for 20 min. The reaction was terminated by adding 2 mL saturated NaCl solution. Four milliliters of chilled diethyl ether was added to each tube to extract benzoylated polyamines. The mixture was vortexed for 20 s then centrifuged for 10 min at 1,500 g_n and 5 $^\circ\text{C}$. The ether fraction was collected and evaporated under a stream of purified nitrogen at 50 $^\circ\text{C}$ using a Reacti-Therm heating block (Pierce Scientific, Rockford, Ill.).

Benzoylated polyamines were resuspended in 0.2 mL chilled methanol and analyzed using a Hitachi dual-pump high-performance liquid chromatography system equipped with a UV detector set at 254 nm, a Brownlee (Applied Biosystem Inc., San Jose, Calif.) RP-18 reverse-phase column (250 $\times$ 4.6 mm), and a column heater. The assay conditions consisted of an isocratic elution of the benzoylated polyamines with 60% methanol in a double-glass distilled water at a flow rate of 0.9 mL $\cdot$ min $^{-1}$ and 30 $^\circ\text{C}$ column temperature.

Conjugated polyamines in the supernatant and in the pellet (suspended in 2 mL of 5% HClO_4) were solubilized by digesting a 0.2 mL subsample of each fraction with 9 N HCl in a flame-sealed

glass ampule at 150 $^\circ\text{C}$ for 8 h (Kushad et al., 1990). The two digestion mixtures were combined, filtered, neutralized, and assayed for polyamines according to the above procedure.

ODC AND ADC ASSAYS. ODC and ADC activities were assayed according to Birecka et al. (1985). Five grams of florets, mesocarp, or seed mass from each tissue, sampling date, and replication were ground in liquid nitrogen into a fine powder with a mortar and pestle and suspended in a buffer containing 50 mM KH_2PO_4 , 2 mM EDTA, 5 mM dithiothreitol, and 50 μM pyridoxal 5-phosphate at 0.3 g $\cdot$ mL $^{-1}$. The mixture was centrifuged at 27,000 g_n and 0 $^\circ\text{C}$ for 30 min, and the supernatant was assayed for ODC and ADC activities. Both enzyme reactions were carried out in 16-mm-diameter borosilicate test tubes capped with double-seal rubber stoppers. A plastic centerwell was suspended in each test tube through the stopper by a stem attached to the centerwell. A 2 $\times$ 3-cm square of Whatman no. 1 filter paper saturated with 150 μL phenethylamine was placed in each well to adsorb CO_2 .

ODC activity was determined by measuring the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine hydrochloride (1.85 TBq $\cdot$ mol $^{-1}$, Amersham Corp., Arlington Heights, Ill.). A 0.2-mL tissue extract and 2.5 Gbq $\cdot$ mol $^{-1}$ of L-[1- ^{14}C]ornithine hydrochloride were combined in a glass test tube with nonradioactive L-ornithine in a total concentration of 1.5 mmol $\cdot$ L $^{-1}$. The test tubes were incubated at 15 $^\circ\text{C}$ for 90 min, then the reaction was terminated by adding 0.5 mL trichloroacetic acid (TCA). The tubes were stored in the dark for ≈ 1 h to allow for maximum CO_2 adsorption into the phenethylamine-soaked filter paper. The filter paper was removed from the well, placed in a scintillation vial containing 8 mL Bio-Safe II (Research Product International Corp. Mount Prospect, Ill.) scintillation fluid, and counted in a scintillation counter (model 1600-TR; Packard Instruments Co., Downers Grove, Ill.).

ADC activity was determined using the same procedure described above for ODC activity except that 2.5 Gbq $\cdot$ mmol $^{-1}$ of L-[U- ^{14}C]arginine (1.85 Tbq $\cdot$ mol $^{-1}$, ICN Biomedical Irvine, Calif.) was used as substrate in a final concentration of 1.25 mmol $\cdot$ L $^{-1}$.

Soluble protein in the extract was determined according to Bradford (1976) using bovine serum albumin as a standard.

Fig. 1. Fresh mass of mesocarp (●) and seed (○) of 'Biscoe' peaches during development. Full bloom (0 sampling time) occurred on 27 Apr. 1996 and fruit were considered commercially mature at 14 weeks after full bloom. Each value represent the mean $\pm$ standard deviation of five replications.

Fig. 2. Ornithine decarboxylase (ODC) (●) and arginine decarboxylase (ADC) (○) activities of mesocarp tissue of 'Biscoe' peaches during development. Full bloom (0 sampling time) occurred on 27 Apr. 1996 and fruit were considered commercially mature at 14 weeks after full bloom. Each value represent the mean $\pm$ standard deviation of five replications.

Results

FRUIT AND SEED GROWTH. Peach mesocarp mass slightly increased during the first 2 weeks AFB then rapidly increased between 3 and 7 weeks (Fig. 1). Thereafter, fruit mass remained steady for ≈ 2 weeks followed by a second phase of rapid growth until maturity.

Seed fresh mass was not determined during the first 2 weeks of fruit growth due to the difficulty of extracting the seeds. Seed fresh mass increased between weeks 3 and 5 AFB then remained relatively unchanged thereafter (Fig. 1).

ODC AND ADC ACTIVITIES. The highest ODC activity was observed in the flower buds (week 0, Fig. 2). Moreover, ODC activity in the flower buds was 4.3-fold that of ADC activity. A rapid decline in ODC activity was observed in the mesocarp up to 7 weeks AFB. However, ADC activity in the mesocarp showed no significant change during the first 6 weeks AFB (Fig. 2). ODC and ADC activities declined slowly in the mesocarp tissue during pit hardening (between week 7 and 9) and during the second phase of rapid growth (Figs. 1 and 2). At full-fruit maturity, ODC and ADC activities were similarly low (Fig. 2).

In seed tissue, ODC activity was highest at 3 weeks AFB (61 $\text{nmol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ protein) then declined as seed size increased (Fig. 3). At 3 weeks AFB, ODC activity was ≈ 2 -fold that of ADC activity. ODC activity continued to decline up to 11 weeks AFB, then gradually increased at 11 to 14 weeks AFB. In contrast, ADC was high at 3 weeks AFB, declined to a low level in week 5, exhibited its highest activity at 8 weeks AFB, then declined. The increase in ADC activity occurred during a period of slow mesocarp growth (Fig. 1).

CHANGES IN POLYAMINES DURING FLESH AND SEED DEVELOPMENT. Free polyamine levels in flower buds were 218, 357, and 535 $\text{nmol}\cdot\text{g}^{-1}$ fresh mass for spermine, putrescine, and spermidine, respectively (Fig. 4A). Polyamines decreased rapidly during the first 2 weeks AFB, followed by an increase in putrescine and spermidine at 2 weeks AFB, and then dropped thereafter (Fig. 4A).

In the mesocarp tissue, conjugated putrescine level increased

slightly during the first 2 weeks AFB, then dropped as the fruit increased in size (Fig. 4B). However, in the same tissue, conjugated spermidine and spermine increased during the first 6 weeks AFB then declined (Fig. 4B). The concentrations of conjugated spermidine and spermine were similar during all stages of mesocarp development, but they were double the concentration of conjugated putrescine after the first 2 weeks of growth (Fig. 4B).

In the mesocarp tissue, total polyamines (free plus conjugated, Fig. 4 A and B) were highest during the first 10 weeks AFB. In flower buds (0 weeks AFB), only 53% of total polyamines were in conjugated form, while in mesocarp tissues conjugated polyamines reached as high as 87% of the sum of all polyamines at 8 weeks AFB (Fig. 4 A and B).

Unlike in the mesocarp tissue, free polyamines levels in the seed did not change between 3 and 7 weeks AFB, except for spermine, which exhibited a slight decline between 3 and 5 weeks AFB (Fig. 5A). Seed tissue contained a higher ratio of free spermine relative to spermidine and putrescine (except between weeks 8 and 10 AFB) than the mesocarp tissue. Spermidine and spermine levels continued to gradually decline; however, putrescine abruptly increased at 8 to 10 weeks reaching a high of 362 $\text{nmol}\cdot\text{g}^{-1}$ fresh mass at 9 weeks AFB (Fig. 5A). Total polyamine levels remained relatively constant after the fruit had reached full fruit maturity (data not shown).

Between 4 and 10 weeks AFB, conjugated spermine increase sharply then slightly declined until fruit maturation (Fig. 5B). Conjugated putrescine and spermidine in the seed also increased (Fig. 5B). Conjugated putrescine increased by $\approx 50\%$ at 8 weeks AFB and then declined (Fig. 5B), while conjugated spermidine showed an inconsistent increase. At week 10, conjugated spermidine and spermine in the seed were 1,169 and 2,148 $\text{nmol}\cdot\text{g}^{-1}$ fresh mass, respectively (Fig. 5B). At week 14, conjugated spermine was 2.4-fold the combined sum of conjugated putrescine and spermidine (Fig. 5B).

In general, total free and conjugated putrescine, spermidine, and spermine concentrations increased as seed development progressed, reaching a maximum level of 4,390 $\text{nmol}\cdot\text{g}^{-1}$ fresh mass at 10 weeks AFB, then decreased during the last 4 weeks AFB (Fig.

Fig. 3. Ornithine decarboxylase (ODC) (●) and arginine decarboxylase (ADC) (○) activities of seed tissue of 'Biscoe' peaches during development. Full bloom (0 sampling time) occurred on 27 Apr. 1996 and fruit were considered commercially mature at 14 weeks after full bloom. ODC and ADC activities were not determined during the first 2 weeks of seed development because it was not possible to extract the seed from the mesocarp. Each value represent the mean $\pm$ standard deviation of five replications.

Fig. 4. Free (A) and conjugated (B) polyamines content of mesocarp tissue of 'Biscoe' peaches during development. Putrescine (■), spermidine (●), and spermine (△). Full bloom (0 sampling time) occurred on 27 Apr. 1996 and fruit were considered commercially mature at 14 weeks after full bloom. Each value represent the mean $\pm$ standard deviation of five replications.

5 A and B). The increase in total polyamines in the seed tissue was mostly in conjugated form ranging from 82% to 97% from early to later stages of development (Fig. 5 A and B).

Discussion

'Biscoe' peaches exhibited a double-sigmoidal growth curve characteristic of stone fruit, figs, and seeded grapes (Westwood, 1978). The initial increase in fruit growth (0 to 7 weeks AFB) has been attributed to cell division, while the second phase of fruit growth (9 to 14 weeks AFB) has been attributed to cell enlargement (Romani and Jennings, 1971). Cessation of mesocarp and seed growth at 7 to 9 weeks AFB is due to lignification of the endocarp (stone) tissue (Romani and Jennings, 1971; Westwood, 1978). Flurkey and Jen (1978) reported an increase in total solids and protein concentration during pit hardening, suggesting that pit hardening is an active growing period. Powell and Pratt (1960) evaluated changes in indoleacetic acid (IAA) during development

of 'Halehaven' peach. They found no change in IAA concentration during the period of pit hardening, suggesting that pit hardening is not an active growth stage.

Polyamine profiles were different between the tissue types and among stages of developments that were studied. Concentration of free putrescine and spermidine were higher in flower buds, declined during the first 2 weeks of fruit development, then increased during weeks 3 and 4. Similar trends in free polyamines were observed in tobacco (*Nicotiana tabacum*) ovaries, citrus flowers, and 'Murcott' mandarins (*Citrus reticulata*) and apples (Biasi et al., 1988; Kushad et al., 1990; Nathan et al., 1984; Slocum and Galston, 1985). Free spermidine in 'Biscoe' flower buds and in the fruit at 2 weeks AFB was higher than putrescine and spermine, while during early stages of fruit growth (3 to 5 weeks AFB) free putrescine was higher than spermidine and spermine.

Fig. 5. Free (A) and conjugated (B) polyamines levels in seed tissue of 'Biscoe' peaches during fruit development. Putrescine (■), spermidine (●), and spermine (△). Full bloom (0 sampling time) occurred on 27 Apr. 1996 and fruit were considered commercially mature at 14 weeks after full bloom. Polyamines were not determined in the seed during the first 2 weeks of development because it was not possible to extract the seed from the mesocarp. Each value represent the mean $\pm$ standard deviation of five replications.

Previous studies on the relationship between polyamines and flower development have indicated that a higher ratio of spermidine to putrescine is required for initiation and development of flower buds (Galston et al., 1990; Harkess et al., 1992). In thin-layer cultures of 'Wisconsin-38' tobacco, exogenous application of spermidine induced flower initiation, while inhibition of spermidine synthesis with cyclohexylamine blocked flower initiation and development (Kaur-Sawhney et al., 1988). Harkess et al. (1992) reported a 2.7-fold higher spermidine than putrescine during initiation and development of *Rudbeckia hirta* flowers. The higher ratio of spermidine to putrescine in 'Biscoe' flower suggests that polyamines are involved in the development of the flower bud. A higher ratio of putrescine to spermidine during early stages of fruit development has been attributed to an increase in cell division (Bacharach, 1973). In peaches, active cell division occurs 2 to 6 weeks AFB (Romani and Jennings, 1971).

In contrast to the mesocarp, seed tissues accumulated higher levels of free spermine than putrescine or spermidine, except during pit hardening, when putrescine concentration and ADC activity increased by 2.5- and 4-fold, respectively (Figs. 4 and 5). The increased synthesis of free putrescine in the seed during pit hardening may have contributed to the increase in conjugated spermidine and spermine and in total polyamines, since spermidine and spermine are synthesized from putrescine.

In plants, putrescine is synthesized via one of two pathways. One pathway involves the synthesis of putrescine through ornithine due to enhanced activity of ODC. This pathway is active during normal growth and development (Slocum and Weinstein, 1990). The second pathway occurs via arginine due to enhanced activity of arginine decarboxylase (Flores et al., 1985). This pathway is active during various types of stresses (Krishnamurthy and Bhagwat, 1989; Richards and Coleman, 1952; Slocum and Weinstein, 1990).

Increase in putrescine synthesis and ADC activity during pit hardening suggest that seed tissues may have been exposed to stress, possibly osmotic. Batjer and Westwood (1958) reported that peach seed growth stops at pit hardening while its dry matter content continued to increase. Polyphenol oxidase has also been shown to increase during pit hardening, which suggests that the tissue is under stress (Flurkey and Jen, 1978). Tiburcio et al. (1986) reported a very large increase in putrescine and ADC activity when cereal leaves were subjected to osmotic stress. It is also possible that the increase in putrescine and ADC activity during pit hardening is related to an increase in gibberellic acid due to embryo development (Jackson, 1968). Perez-Amador and Carbonell (1995) reported increased putrescine and ADC activity in pea ovary treated with gibberellic acid.

Interestingly, however, decreased growth of mesocarp tissue during pit hardening did not enhance the synthesis of putrescine or ADC activity or cause a major shift in the profile of the polyamines pool, which suggests that polyamine synthesis in the seed and the mesocarp are independently regulated. Jackson (1968) studied gibberellin levels during peach seed growth, pit hardening, and expansion of the mesocarp. He concluded that increased hormonal levels in specific parts of the fruit occur only as a function of the growth of that particular tissue, thus detracting from the concept of a strong interrelationship between seed growth, pit hardening, and mesocarp growth.

In general, the concentration of total spermine in the seed increased during sampling, reaching its highest level at 10 weeks AFB. Sinska and Lewandowska (1991) reported that spermine concentration in dormant apple seeds was twice as high as in nondormant seeds, suggesting a role for spermine in seed dor-

mancy. Cochran et al. (1961) reported that seeds of most peach cultivars are dormant after harvest, but they will germinate within 100 to 120 d of storage at 4.5 °C.

In conclusion, polyamine profiles in peach mesocarp tissue exhibited similar patterns to those previously reported in apple and avocado (Biasi et al., 1988; Kushad et al., 1988). Seed tissue exhibited a burst of free putrescine and conjugated putrescine, spermidine, and spermine during pit hardening. The increase in putrescine and ADC activity in the seed during pit hardening were similar to those reported during various stresses. Increased putrescine synthesis in the seed during pit hardening did not affect the level of polyamines in the mesocarp tissue, suggesting that polyamine are independently regulated in these tissue.

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