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Carbohydrate metabolism of vegetative and reproductive sinks in the late-maturing peach cultivar 'Encore'

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Summary Activities of NAD '-dependent sorbitol dehydrogenase (SDH), sorbitol oxidase (SOX), sucrose synthase (SS), acid invertase (AI), and neutral invertase (NI) in 'Encore' peach (*Prunus persica* L.) fruits and developing shoot tips were assayed during the growing season to determine whether carbohydrate metabolizing enzymes could serve as indicators of sink strength. In fruit flesh, SS activity was detected during Stage I of growth, when cells were actively dividing, and SDH activity was detected during Stage III, when cells were actively enlarging. Acid invertase activity was detected during Stage I and showed a closer correlation with relative increase in fruit weight during the growing season than SS activity. During seed filling and pit hardening (Stage II), when relative fruit growth rate was slowest, activities of carbohydrate metabolizing enzymes in fruit flesh were not detectable. No SOX activity was detected during Stages I and II. The highest sucrose content occurred near the end of fiuit development when the activities of sucrose metabolizing enzymes were low. In developing shoot tips, the sorbitol:sucrose ratio was 2 : 1 (w/w) and SDH activity was low at the beginning and end of the season when vegetative growth was slowest. The sorbitol:sucrose ratio changed to 1:1 (w/w) along with an increase in SDH activity in shoot tips during the mid-growing season. In 'Nemaguard' peach, SDH exhibited higher activity in root tips than in other organs. Among the sorbitol- and sucrosemetabolizing enzyme activities, only SDH activity was positively correlated with shoot growth in 'Nemaguard' plants.

Keywords: acid invertase, fruit, growth, neutral invertme, shoot tips, sorbitol dehydrogenase, sorbitol oxidase, sucrose, sucrose synthase.

Introduction

In general, the strength of a sink, defined as the ability to attract photosynthate, is determined not only by the sink, but also by the source, pathway, and other sinks (Minchin and Thorpe 1996). Factors such as sink size, time of initiation relative to other sinks, location, and distance from the source are important determinants of sink strength in the whole plant (Ho 1980, Bangerth and Ho 1984, Wardlaw 1990).

In a fruit tree at harvest, for example, high crop loads can account for 50% of total dry matter production. Fruits, therefore, represent strong sinks and can compete successfully for assimilates with vegetative organs (Weinberger 193 1, Martin et al. 1964). The double-sigmoid growth pattern of peach fruit (Chalmers and Van den Ende 1975) indicates variations in sink strengtb at different growth stages. Thus, Stage I is characterized by active cell division and hence rapid growth, Stage II is characterized by relatively slow growth during seed filling and pit hardening, and Stage III is characterized by fruit swelling as the cells enlarge (Ognjanov et al. 1995).

Methods of measuring sink strength, such as growth rate, net accumulation of dry matter, or the rate of synthesis of carbon reserves, do not consider the proportion of assimilate used for respiration and therefore underestimate the potential of a sink to receive and metabolize assimilate (Ho 1988). A more appropriate estimate of a sink's strength is given by the sum of its net carbon gain and respiratory carbon loss (Ho 1988). Because of the predominance of sucrose as the translocated form of carbon in plants, Sung et al . *(1989a)* found that the activity of sucrose cleavage enzymes in sink tissues provided a reasonable measure of sink strength.

In plants where sucrose is not the major form of translocated carbon, enzymes responsible for the metabolism of other translocated assimilates could serve as indicators of sink *strength. In* peach, and *in many* species of the *Rosaceae,* sorbitol is the major photosynthetic product and the main form of translocated carbon along with sucrose (Bieleski 1982). Sorbitol:sucrose (w/w) ratios ranging from 1.3 :1 to 4.2 :1 have been reported for mature leaves of peach (Escobar-Gutierrez and Gaudillère 1994), and a ratio of 3 :1 was determined in apple bark (Webb and Burley 1962). Although sorbitol oxidase (SOX), which converts sorbitol to glucose (Yamaki 1980), seems to play a minor role during the early stages of fruit growth in apple (Yamaki and Ishikawa 1986), in peach fruit of the cultivar 'Hakuto,' SOX represents the main enzyme of sorbitol cleavage with maximum activity (0.9 nmol mi n^{-1} g^{-1} fresh weight) occurring during the early stages of fruit development (Moriguchi et al. 1990). However, the same authors concluded that SOX activity alone could not explain all of the *in vivo* sorbitol interconversions in peach fruit.

Seasonal changes in NAD +-dependent sorbitol dehydrogenase (SDH), which catalyzes the oxidation of sorbitol to fructose and is found primarily in sink tissues (Loescher 1987), have been studied in fruits and mature apple leaves (Yamaki and Ishikawa 1986). In addition, leaf developmental stage has been correlated to SDH activity in apple (Loescher et al. 1982) and peach (Merlo and Passera 1991). Although sucrose metabolism has been studied in peach fruit (Moriguchi et al. 1990, Vizzotto et al. 1996), the effects of sorbitol metabolism on growth of reproductive and vegetative sinks in peach have not been studied.

We hypothesized that SDH activity could serve as an indicator of sink strength in peach. To test our hypothesis, seasonal changes in activities of both sorbitol- and sucrosecleavage enzymes were measured and related to the growth and development of reproductive and vegetative peach sinks. Amounts of carbohydrates and starch were also determined in all samples. Correlations between shoot growth rate and the activities of sorbitol- and sucrose-metabolizing enzymes were tested in a separate experiment.

Materials and methods

Fruits and actively growing shoot tips were collected from 6-year-old 'Encore' peach (*Prunus persica* L.) trees. Plants were grown in the field on the rootstock 'Lovell,' at a spacing of 2 x 7 m. The soil was a Cecil sandy clay loam. Shoot tips consisted of the apical meristem and all of the folded leaves and varied in length from 2 to 3 cm and varied in fresh weight from 20 to 60 mg per shoot tip. About 7 O-80 shoot tips and a variable number of fruits, depending on size, were collected every other week starting 7 days after initial bud break and 24 days after initial blooming, respectively. Samples for each measurement date were harvested within a l-h period (usually in the morning) to reduce variation caused by daily fluctuations in plant metabolism. Samples were transported from the field to the laboratory in plastic bags on ice. A subset of the samples was assayed immediately for SDH, and the remaining samples were frozen at -20 °C for subsequent assays of sucrose-cleavage enzymes and analysis of carbohydrates.

In September, when vegetative growth had stopped in the field, shoot tips from l-year-old rooted cuttings of 'Nemaguard' peach were used to study the correlations between elongation rate and specific activities of sucrose and sorbitol-metabolizing enzymes. Plants were grown hydroponically in a greenhouse as described by Rieger and Scalabrelli (1990). Root tips of the same plants were used to determine enzymatic activities. Both experiments were conducted in Athens, GA (35° N, 85° W).

Enzyme extraction and assays

For shoot tips, SDH was extracted by homogenizing 1 g of tissue in 10 ml of Tris extraction buffer consisting of 0.1 M Tris-HCl buffer (pH 9 at 25 °C) containing 8% (v/v) glycerol. Because 2-mercaptoethanol in buffer is unstable, it was added (20 mM) immediately before each extraction. Tween 20 (0.1%, v/v) and polyvinylpolypyrrolidone (PVPP; 1%, w/v) were added during grinding. For the 'Nemaguard' experiment, a single shoot tip (0.0 6-0.1 g) was homogenized in 5 ml of Tris extraction buffer. For tits, SDH was extracted by homogenizing 2.5 to 5 g, depending on the protein content, of peeled and cored tits in 6 ml of Tris extraction buffer. Sucrose synthase (SS), acid invertase (AI), neutral invertase (NI), SOX, and UDP-glucopyrophosphorylase were extracted from both fruits and shoot tips in 0.2 M Hepes-NaOH buffer (pH 7.5 at 25 °C) containing 10 mM dithiothreitol (DTT), 3 mM Mg-acetate, and 6% (v/v) glycerol. Tween 20 (0.1%, v/v) and PVPP (1%, w/v) were added during grinding. Amounts and proportions of tissues and buffer were similar to those used for SDH. Sorbitol- and sucrose-metabolizing enzymes were extracted from 0.3 g of 'Nemaguard' root tips (terminal 2 cm) in 5 ml of the appropriate extraction buffer. In all cases, the tissue was ground in buffer and sand with a chilled (2-4 °C) mortar and pestle. The homogenate was filtered through a layer of miracloth and centrifuged at 3000 g for 15 min. The supematant was desalted by chromatography through a Sephadex G-25 (medium) column at 4 °C. In all cases, there were three replicates, where a replicate constituted one extraction for each set of enzymes.

Sorbitol dehydrogenase was assayed using 0.1 ml of desalted extract, 0.1 M Tris-HCl buffer (pH 9.5 at 25 °C), and 1 mM NAD^+. The assay mixture was incubated at 25 °C for 5 min and the reaction was started by adding 300 mM sorbitol (1 ml final volume). Enzymatic activity was determined spectrophotometrically at 340 nm (Spectronic 21-D, Milton Roy, Rochester, NY) over a 5-min period. Sucrose synthase, AI, NI and UDP-glucopyrophosphorylase were assayed as described by Xu et al. (1989). Sucrose synthase was determined spectrophotometrically at 340 mn at 25 °C in an assay containing 100 mM sucrose, 0.5 mM UDP, and 1 mM PPi as substrates and phosphoglucomutase (1 U) and *Leuconostoc* glucose-6-phosphate dehydrogenase (1 U) as coupling enzymes. The assay for UDP-glucopyrophosphorylase consisted of UDP-glucose (1 mM), 1 mM PPi, and the same coupling enzymes. Acid invertase and NI were assayed with 25 mM sucrose at pH 5 and 100 mM sucrose at pH 7, respectively, whereas SOX was assayed with 400 mM sorbitol at pH 4. After a 1 5-20 min incubation at 25 °C, the reaction was stopped by boiling for 10 min. The AI and SOX reaction mixtures were neutralized before boiling. The glucose formed was measured using hexokinase (1 U) and *Leuconostoc* glucose-6-phosphate dehydrogenase (1 U). In all enzyme assays, activities were proportional to the amount of extract and time.

Protein content was determined by the method of Bradford (1976). Enzyme specific activity was expressed as nanomoles of NADH produced per minute per milligram of protein or per gram of tissue fresh weight. In fruits, total enzyme activity was expressed as nanomoles of NADH produced per minute per fruit.

Sorbitol and nonstructural carbohydrates

Nonstructural carbohydrates were quantified in freeze-dried tissues by gas chromatography (Rieger and Marra 1994). After

homogenization of 100 mg of shoot tip tissue or 75 mg of fruit tissue in 80% methanol containing 0.22 mg m l^{-1} of phenyl- β-D-glucopyranose as an internal standard extracts were centrifuged for 3 min at 3900 g. One or 2 ml of supematant, which constituted the soluble carbohydrate fraction, was pipetted into vials and stored at 4 °C. The pellet was washed and centrifuged twice with 80% methanol. Ten ml of deionized water was added and tubes were incubated at 100 °C for 1 h to gelatinize starch. Afterwards, 5 ml of internal standard (xylitol, 1 mg in 5 ml H_2O), 1 ml of acetate buffer, and 0.1 ml of amyloglucosidase enzyme solution (19.7 U) were added and tubes were incubated at 55 °C for 48 h to hydrolyze the starch to glucose. Tubes were centrifuged for 3 minutes at 3900 g and a l-2 ml aliquot of the supematant was stored in a freezer for starch analysis. Subsequently, 0.1 ml samples were dried in GC vials and derivatized for injection on a gas chromatograph (Hewlett Packard 5890A, Avondale, PA).

Growth analysis

Fruits collected for determination of enzymatic activities (6 to 20, depending on their size) were individually weighed and a mean fresh weight (FW) per sampling date was reported in the growth curve. Fruit relative growth rate (RGR) was calculated on the basis of mean fresh weight according to the following formula:

$$\mathrm{RGR} = \frac{\ln \mathrm{FW}_2 - \ln \mathrm{FW}_1}{T_2 - T_1}, \qquad (1)$$

where FW is expressed in grams and T is number of days after initial blooming.

About 12 to 15 shoots of 'Nemaguard' plants were labeled and measured in length twice, at an interval of 2-3 days. The net increase in length was divided by the number of days between the two measurements and growth rate (mm da y^{-1}) calculated. Each shoot tip represented a sample and only three tips from shoots growing at different rates were harvested for assay.

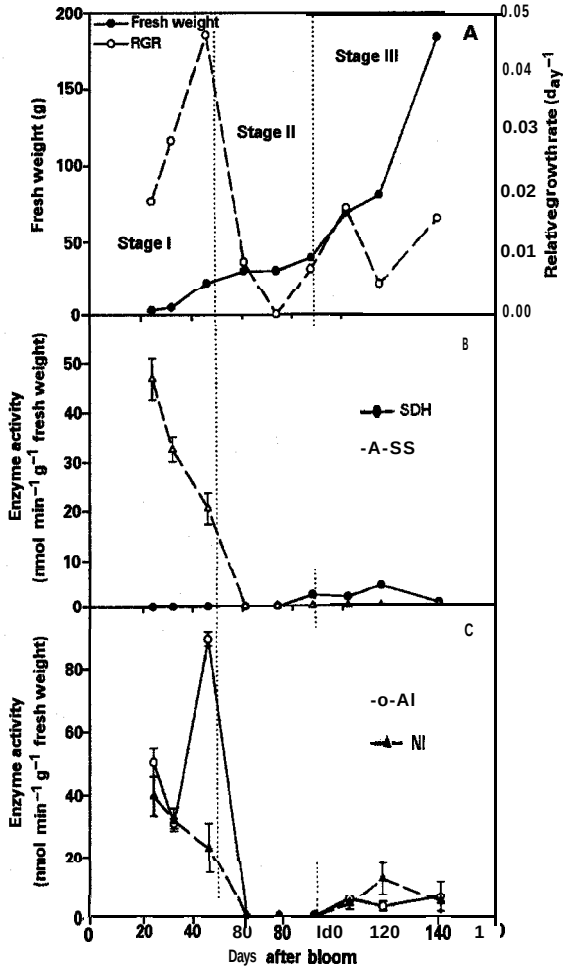

Figure 1. 'Encore' peach fiuit growth curve and relative growth rate (A). Activities of sorbitol dehydrogenase (SDH), sucrose synthase (SS), acid invertase (AI), and neutral invertase (NI) in fruit flesh expressed on a g fresh weight basis (B and C). Error bars represent standard errors of the means (n = 3). Error bars smaller than symbol size are not shown.

Results

Fruit

The fruit growth curve followed the double-sigmoid pattern that is characteristic of stone fruits (Figure 1A). For the first 50 days after bloom, cell division was rapid and RGR was high (Stage I). During the subsequent 40 days, when seed filling and pit hardening occurred, there was only a small increase in fruit mass (Stage II). Fruit growth resumed about 90 days after bloom, when the cell enlargement phase took place (Stage III), and ended about 138 days after bloom (commercial ripening).

There were no apparent differences in the seasonal trend of the measured enzyme activities in peach fruits between protein and fresh weight basis. Thus, only fresh-weight-based specific enzyme activities are presented here. Sucrose synthase activity was detected only during Stage I of fruit growth (Figures 1B and 2A), and a positive correlation with growth (r = 0.957) was found only when activity was expressed on a per fruit basis (Figure 2A), not on a mg protein (data not shown) or g fresh weight basis (Figure 1B). In contrast, SDH activity was detected only during Stage III of fruit growth (Figures 1B and 2A), and its total activity was inversely correlated with fruit growth (r = -0.807; Figure 2A). Both AI and NI activities were present during Stages I and III of fruit growth (Figures 1 C and 2B). Furthermore, AI specific activity tracked fruit RGR over the entire growing season with r values of 0.888 and 0.907 when expressed on a fresh weight and protein basis, respectively. No sorbitol- or sucrose-metabolizing enzyme activity was detected in fruit flesh during the seed tilling and pit hardening (Stage II). Activity of UDP-glucopyrophosphorylase during Stage II was about one-seventh (3 10 nmol mi n^{-1} mg^{-1} protein) that during Stages I (2400 nmol mi n^{-1} mg^{-1} protein) and III (2200 nmol mi n^{-1} mg^{-1} protein), but it was always much higher than all of the other enzymatic activities

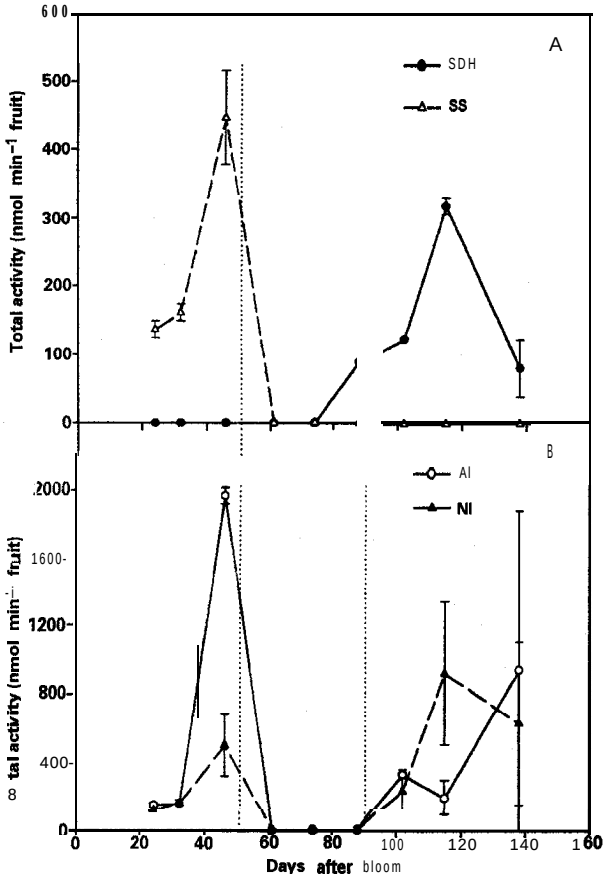

Figure 2. Total activity per 'Encore' peach fruit of sorbitol- and sucrose-metabolizing enzymes during fruit development. Abbreviations: SDH = sorbitol dehydrogenase; SS = sucrose synthase; AI = acid invertase; and NI = neutral invertase. Error bars represent standard errors of the means (n = 3). Error bars smaller than symbol size are not shown.

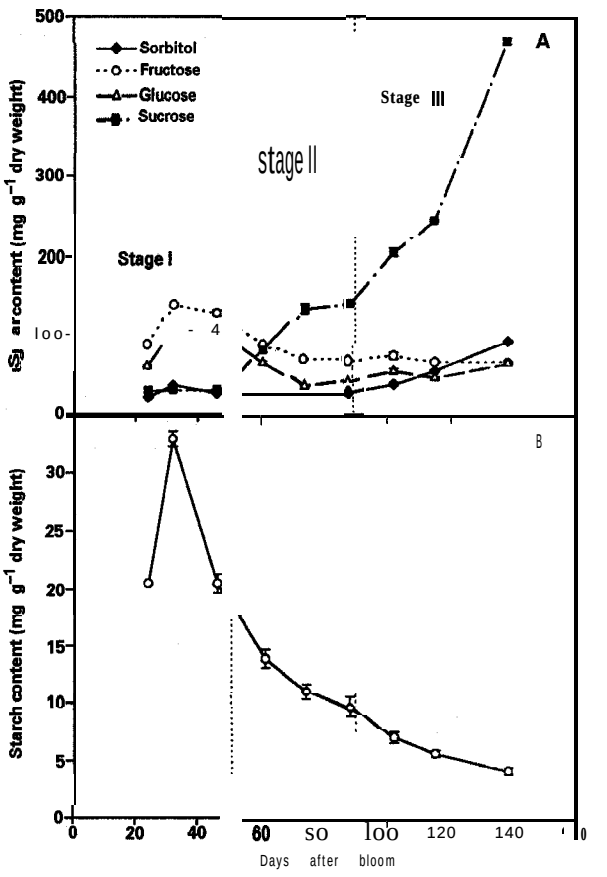

Figure 3. Contents of soluble carbohydrates (A) and starch (B) in 'Encore' peach fruit flesh during development. Error bars represent standard errors of the means (n = 3). Error bars smaller than symbol size are not shown.

measured, and thus did not limit the apparent SS activity. No SOX activity was detected during Stages I and II, indicating that SOX did not play a role in fruit tissue development when SD3 was not present.

Sorbitol content in fruits was consistently low during most of the season, and only increased slightly during Stage III (Figure 3A). Sucrose content was low at the beginning of fruit development, but increased progressively during fruit development and was 4–6-fold higher than sorbitol content by the end of Stages II and III. Fruits contained more fructose than glucose during the entire season. Hexose content was highest during Stage I, when sucrose was low, and declined to a constant low value by Stage III. After an initial increase, the starch content declined progressively throughout the growing season (Figure 3B).

Shoot tips

Sorbitol dehydrogenase activity in developing shoot tips followed a quadratic response (P = 0.022) with a peak around Day 54 after bud break (Figure 4). Sucrose synthase activity followed closely the pattern of SDH activity for the first 98 days, but unlike SDH, it did not decline during the last 25 days of vegetative growth (Figure 4). Both AI and NI activities were relatively high during the entire growth period, although NI activity declined after the first 98 days (Figure 4).

The content of sorbitol was almost double that of sucrose at the beginning (until 15 days after bud break) and at the very end (Day 122) of the growing season, whereas sucrose and sorbitol maintained similar contents and temporal patterns during the middle part of the growing season (Figure 5A). Fructose and glucose contents remained low during most of the growing season (Figure 5A). The starch content, after an initial rapid drop, increased at mid-season, then gradually declined until terminal buds had set (Figure 5B).

Root tips

No SS activity was detected in tips of actively growing roots of 'Nemaguard' rooted cuttings grown hydroponically. In contrast, root-tip SDH activity was relatively high (244 nmol min^{-1} g^{-1} fresh weight and 54 nmol min^{-1} mg^{-1} protein) compared to SDH activity in shoot tips (145 nmol min^{-1} g^{-1} fresh weight and 3.4 nmol min^{-1} mg^{-1} protein) of the same plants and 'Encore' fruits (5 nmol min^{-1} g^{-1} fresh weight and 8 nmol min^{-1} mg^{-1} protein). In root tips, SDH activity was 150% of AI activity and 660% of NI activity when activities

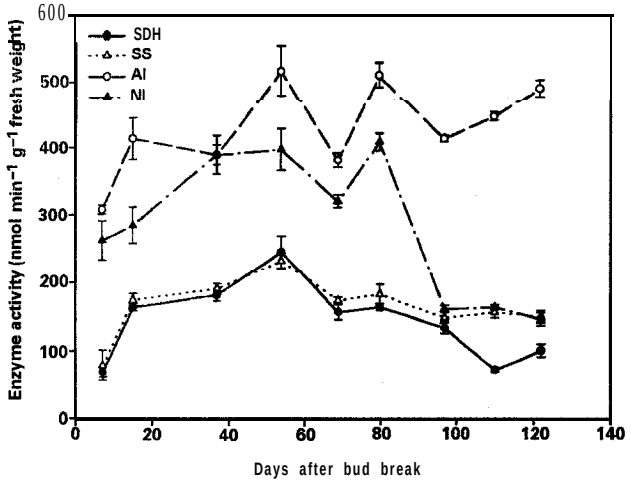

Figure 4. Specific activities of sorbitol- and sucrose-metabolizing enzymes in developing shoot tips of 'Encore' peach trees harvested throughout the growing season. Abbreviations: SDH = sorbitol dehydrogenase; SS = sucrose synthase; AI = acid invertase; and NI = neutral invertase. Error bars represent standard errors of the means ($n = 3$). Error bars smaller than symbol size are not shown.

were expressed on a gram fresh weight basis, but only 26% of AT activity and 104% of NI activity when expressed on a mg protein basis. However, in other organs, Al activity was always two to ten times higher than SDH activity, regardless of the basis used to express activity.

Correlation between shoot elongation rate and enzyme activities

Results of the preliminary experiment with a shoot tip from a 'Nemaguard' plant are reported as a regression plot where the slope of the lines describes the relationship between shoot growth rate and activities of sorbitol- and sucrose-metabolizing enzymes (Figure 6). Only the slope of the regression line for SDH activity *versus* growth rate was significantly positive ($P = 0.042$) whereas the remaining slopes were either negative (SS) or zero (AI and NI).

Discussion

In the late-maturing peach cultivar 'Encore,' sucrose- and sorbitol-metabolizing enzymes seem to have different roles during the various stages of fruit growth. Acid invertase was active during Stage I, when fruit growth was characterized by rapid cell division, and was closely correlated with RGR ($r = 0.9$) during the entire season (Figures 1 and 2). Although SS activity was detected during Stage I (Figures 1 and 2), the correlation between SS activity and RGR was low compared with the correlation between AI and RGR. Neither AI nor SS activity was detected during Stage II of fruit development. The developmental patterns of peach fruit AI and SS activities reported here are similar to those of 'Redhaven' peach, except that 'Redhaven' fruits contained some AI and SS activities during Stage II, and SS activity was always higher than in the present study (Vizzotto et al. 1996). In the cultivar 'Hakuto,' SS activ-

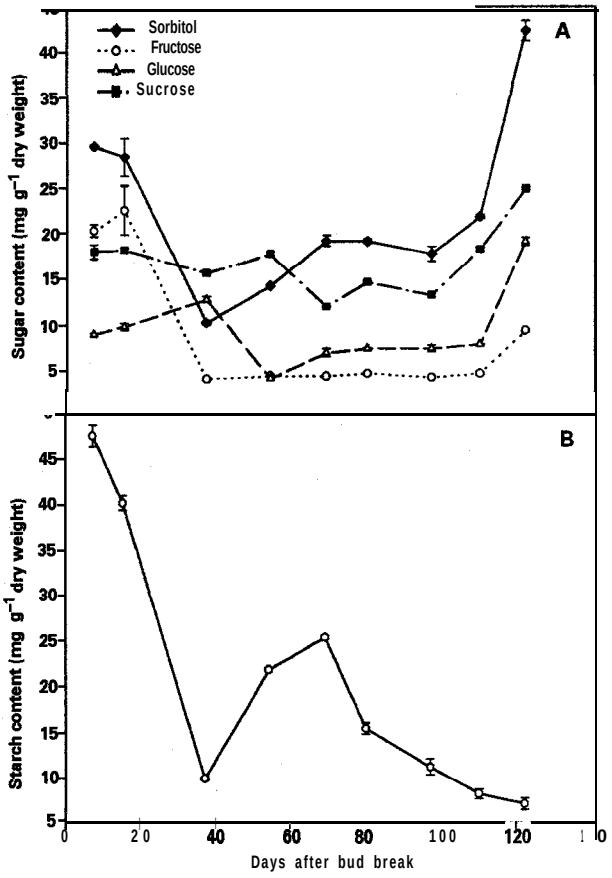

Figure 5. Contents of soluble carbohydrates (A) and starch(B) in shoot tips of 'Encore' peach trees harvested throughout the growing season. Error bars represent standard errors of the means ($n = 3$). Error bars smaller than symbol size are not shown.

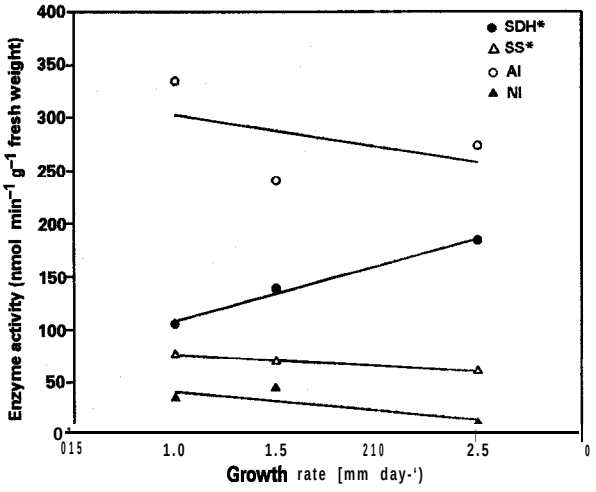

Figure 6. Correlations between growth rate and specific activities of sorbitol- and sucrose-metabolizing enzymes of shoots of 'Nemaguard' peach. Abbreviations: SDH = sorbitol dehydrogenase; SS = sucrose synthase; AI = acid invertase; and NI = neutral invertase. Each point represents one replicate. Lines in the graph represent linear regressions and an asterisk indicates statistical significance of the slopes ($P \leq 0.05$).

ity was also detected during Stage III (Moriguchi et al. 1990). The absence of activity of sucrose-metabolizing enzymes in fruit flesh during Stage II in this study probably was not caused by the presence of inhibitors or a failure to extract the fruit tissue proteins, because UDP-glucopyrophosphorylase activity was detected during Stage II. Because peach fruits are actively filling seeds and hardening pits during Stage II, the disappearance of sucrose metabolizing enzymes probably indicates that both AI and SS are inducible enzymes whose activities are closely correlated with peach fruit growth. Acid invertase activity is also high during the early stages of rapid growth in melon fruits (Iwatsubo et al. 1992), in immature juice sacs of satsuma mandarin (Kato and Kubota 1978), grape berries (Ruffner et al. 1995), and in 'Hakuto' peach tit (Moriguchi et al. 1990).

In addition to a role in fruit growth, AI may have a role in determining the sugar composition of 'Encore' peach fruit. Acid invertase was most active during Stage I, when sucrose content was lowest and reducing sugars highest, whereas it was relatively low during Stage III, when sucrose accumulated in large amounts (Figures 1 and 3). Similar results were obtained by Vizzotto et al. (1996) in 'Redhaven' peach fruit, whereas Moriguchi et al. (1990) concluded that sucrose accumulation in 'Hakuto' peach fruit was mainly the result of SS activity. In the present study, SS activity, which is generally believed to operate primarily in the direction of sucrose degradation *in vivo*, could be associated with the temporary accumulation of starch during Stage I (Figures 1 and 3) as reported in apple fruit (Bet-titer et al. 1997) and tomato fruit (Wang et al. 1993).

Sorbitol dehydrogenase activity was detected during the final swelling of fruits, but was not correlated with RGR (Figures 1 and 2). In contrast, Yamaki and Ishikawa (1986) detected SDH activity during the entire fruit development period of apple fiuit, whereas in other studies of apple fruit, SDH activity was observed only at the end of the season (Berüter 1985, Yamaguchi et al. 1996). Although SDH activity was generally lower than the activities of AI and NI during Stage III, SDH was the main sorbitol cleavage activity in 'Encore' fruits, because no SOX activity was detected during fruit development. In 'Hakuto' peach fruits, Moriguchi et al. (1990) found that SOX was the main enzyme of sorbitol cleavage, although its activity was low (0.03 -0.9 nmol min^{-1} g^{-1} fresh weight).

Because no activity of sorbitol-metabolizing enzymes was detected during Stage I, the role of sorbitol in the early stages of fruit growth is unclear. However, a consistently low sorbitol content expressed on a dry weight basis during Stages I and II could be partly explained by preferential unloading of sorbitol into vegetative sinks close to the source, such as young leaves and cambium (Moing et al. 1992).

In developing shoot tips, SS and SDH activities reached their maximum in mid-growing season, when growth and enzymatic activities in fruits were lowest (Figures 1, 2 and 4). During this period, trees were probably allocating more sugars to vegetative sinks than to reproductive sinks. The extra carbon partitioned to shoots was not only invested in growth, but also in storage, as indicated by the temporary increase in starch content 4 O-80 days after bud break (Figure 5B). Therefore, the general assumption of fruits outcompeting vegetative sinks for carbon does not apply to peach fruits, in which there is a complex temporal pattern of competition for carbohydrates between fruits and shoots.

Both shoot tip SS and SDH activities were relatively low at the beginning of the season, when shoot growth was slow. However, the decrease in vegetative growth at the end of the season was accompanied by a decline in SDH activity, but not in SS activity. Acid invertase did not follow any specific pattern and may function as a maintenance enzyme, as reported in other species (Sung et al. 1989 a, 19893). Amounts of sorbitol and sucrose were similar during most of the growing season; however, a 2 :1 ratio of sorbitol:sucrose was observed both at the beginning and end of the season when shoot growth rate and SDH activity were low. This pattern provides further evidence that sorbitol is the main source of carbon used for growth in vegetative sinks of peach. Moreover, SDH activity in actively growing roots of 'Nemaguard' plants was higher than SDH activity in shoot tips of the same plants, in fruits of 'Encore,' and higher than AI and NI activities in the same roots, implying that sorbitol may have a predominant role in the growth and metabolism of roots.

When enzymatic activities were plotted against elongation rate of 'Nemaguard' shoots, only SDH activity showed a positive correlation (Figure 6). These preliminary data together with our finding of a positive correlation between SDH activity and vegetative growth in 'Encore' shoots indicate that SDH could serve as an indicator of sink strength in vegetative organs.

We conclude that the metabolism of peach sinks is complex. The proportions of the two major forms of carbon, sorbitol and sucrose, change depending on the stage of growth and the organ. It is postulated that sucrose is the major carbon form used for fruit growth, whereas sorbitol has a predominant role in vegetative growth.

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