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Full Length Research Paper

Changes in the organic acid content and related metabolic enzyme activities in developing 'Xinping' pear fruit

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Changes in the content of citric acid and malic acid and the activities of enzymes involved in the metabolism of these two organic acids, including citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (IDH), phosphoenolpyruvate carboxylase (PEPC), NAD-malate dehydrogenase (NAD-MDH) and NADP-malic enzyme (NADP-ME) were examined during the process of fruit development in 'Xinping' pear. The citric and malic acid content exhibited an overall increasing trend in developing fruit. The activities of CS, NAD-IDH and NAD-MDH showed an overall increasing trend and the activities of Cyt-ACO, PEPC and NADP-ME exhibited an overall decreasing trend in developing fruit. The correlation analysis of the change in the organic acid content and related metabolic enzyme activities during the development of the fruit revealed that CS, ACO, IDH, PEPC, NAD-MDH and NADP-ME are considered important enzymes that affect the synthesis of citric acid and malic acid in pear fruits.

Key words: Pear, fruit, development, organic acid, metabolic enzyme, correlation.

INTRODUCTION

Citric acid and malic acid are major organic acids in pear fruits (Arfaioli and Bosetto, 1993; Hudina and Stampar, 2000) and their content is an important factor that affects the quality of the fruit. Organic acids accumulate during the process of fruit growth and are consumed as respiratory substrates in glycolysis and in the tricarboxylic acid cycle; in addition, the organic acids are utilized as gluconeogenesis substrates during the fruit ripening process. During fruit development, changes in organic acid content are closely related to changes in the corresponding enzyme activities. Related research studies have indicated that citrate synthase (CS) (Yamaki, 1990; Wen et al., 2001) aconitase (ACO), isocitrate dehydrogenase (IDH) (Sadka et al., 2000a) are important enzymes that affect the metabolism of citric acid in fruit; the phosphoenolpyruvate carboxylase (PEPC) (Wen et al., 2001), malate dehydrogenase (MDH) (Miller et al., 1998) and malic enzyme (ME) (Ruffer et al., 1984) are important enzymes that affect the metabolism of malic acid in fruit. The increased citric acid content in lemon fruits has been correlated to an increase in CS activity (Bruemmer et al., 1977). However, it has also been reported that CS activity does not show a correlation with changes in the citric acid level in citrus fruits (Canel et al., 1996; Hirai and Ueno, 1977). A study by Kudo et al. (2002) indicated that there is no correlation between the CS activity and the accumulation of citric acid during the development of Japanese summer orange (*Citrus natsudaidai*) fruit. Sadka et al. (2001) have discovered that the differences in the accumulation of organic acid in acidless and acid-containing citrus varieties were not correlated with mitochondrial CS activity. Therefore, fruits of different cultivars exhibit great differences in the utilisation of the enzymes involved in organic acid metabolism. Little research has been reported on the relationship between the accumulation of organic acids and dynamic changes in the organic acid metabolism-related enzymes in pear fruits. We examined the changes in the accumulation of citric and malic acid, as well as the activities of their related metabolic enzymes during the development of pear fruit using the 'Xinping' pear (*Pyrus bretschneideri* Rehd.) cultivar; this study aimed to investigate the mechanism of the under

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lying organic acid accumulation in pear fruits and identify the key enzymes that participate in organic acid accumulation in pear fruits. In addition, this study will provide a foundation for research on organic acid metabolism and its regulatory mechanism at the molecular level.

MATERIALS AND METHODS

The present study was conducted during the fruit-growing season in 2009. The tested materials were from five-year-old 'Xinping' pear plants, with a rootstock of the sorb tree (*Pyrus ussuriensis* Maxim.), grown in the pear experimental field at the Liaoning Institute of Fruit Science located in Xiongyue Town, Yingkou City, Liaoning Province (40°10' N, 122°09' E, 20.4 m above sea level). The study site has a typical temperate, continental monsoon climate, with an annual average temperature of 9.4°C, annual average rainfall of 614.4 mm, annual average relative air humidity of 66%, hot (average 23.3°C) and short (approximately 80 days) summers and cold (average -6.23°C) and long (approximately 130 days) winters. In 2009, the average temperature was 9.7°C, the average rainfall was 529.1 mm and the average relative air humidity was 63%. The soil used in the study was sandy loam soil, with a pH of 6.28, a soil organic matter content of 0.54% ($w.w^{-1}$), total nitrogen content of 0.06%, phosphorus content of 0.05% and an exchangeable potassium content of 1.82%. The samples were collected from 46 plants of 'Xinping' pear, which were planted with a row spacing of 4 m × 3 m, and had spindle-shaped crowns. All of the plants were grown in accordance with local management standards, including pruning, fertilising and pest control. Samples were collected once every 15 days after the full-bloom stage until the fruits ripened. The fruits were considered ripe when the skin colour changed from green to yellow, and the seed colour from white to dark brown or black. Five fruits were collected at each sampling. The samples were randomly collected at 10 a.m. from the periphery of the upper canopy and the upper part of the fruit-bearing branches of trees with similar growth. The outer skin and the core were removed from the collected fruits, and the leftover fleshy tissue of the 5 fruits was cut into small pieces and mixed together. Approximately 10 g of each sample was placed in a freezing vial, treated with liquid nitrogen and stored at -70°C until use.

The organic acid content was measured according to the method published by Nisperos-Carriedo et al. (1992). Samples were accurately measured, and 1.00 g of the fleshy tissue of the pear fruit was homogenised in 5 ml of ice-cold 0.2% metaphosphoric acid and centrifuged at 10,000 × g for 15 min. The precipitate was extracted again with 4 ml of 0.2% metaphosphoric acid. The supernatants from both extractions were combined, and the volume was fixed at 10 ml. The combined supernatant was then passed through a 0.45 μm filter before measurement. Each sample was measured in triplicate. A Dionex U-3000 HPLC system was used for the organic acid measurements, with a UV/VIS detector and XB-C_{18} (4.6 mm × 250 mm) column. The mobile phase consisted of a 0.2% metaphosphoric acid aqueous solution, the flow rate was 1 $ml.min^{-1}$, the column temperature was 35°C, the injection volume was 10.0 μl and the detection wavelength was 210 nm. Among the used reagents, the metaphosphoric acid was analytical grade and the citric acid and malic acid were both of chromatographic grade standards and provided by Sigma-Aldrich.

The enzymes were extracted following the methods published by Hirai and Ueno (1977) and Luo et al. (2003). Two grams of each sample of the fleshy tissue of the pear fruit was placed in a prechilled mortar, followed by the addition of 2 ml grinding buffer (0.2 $mol.L^{-1}$ Tris-HCl buffer (pH 8.2), 0.6 $mol.L^{-1}$ sucrose and 10 $mmol.L^{-1}$ erythorbate) and homogenisation in an ice bath. The homogenate was centrifuged at 4000 × g for 20 min at 4°C. The supernatant was collected, at a fixed volume of 5 ml. Two millilitres of the supernatant was centrifuged at 15000×g for 15 minutes at 4°C. The volume of the resultant supernatant was then fixed at 4 ml using the extraction buffer (0.2 $mol.L^{-1}$ Tris-HCl, pH 8.2, 10 $mmol.L^{-1}$ erythorbate and 0.1% Triton X-100) to obtain cytosolic aconitase (Cyt-aconitase). The volume of the resultant precipitate was fixed at 2 ml using the extraction buffer to obtain the mitochondrial aconitase extract (Mit-aconitase, ACO) and the NAD-isocitrate dehydrogenase extract (NAD-IDH). The leftover 3 ml of supernatant from the initial homogenisation was mixed with 3 ml of extraction buffer, which was used for the measurement of NAD-malate dehydrogenase (NAD-MDH) and NADP-malic enzyme (NADP-ME). Subsequently, 4 ml of the mixture was dialysed against a large amount of extraction buffer at 4°C overnight to obtain the PEPC extract and the citrate synthase extract (CS).

Enzymatic activities were measured using the methods published by Hirai and Ueno (1977) and Luo et al. (2003). The reaction volume was 3 ml, and the UV absorbance was measured using a UV-2450 spectrophotometer immediately following the addition of the substrate. Changes in the UV absorbance were recorded over a 3-min scan with an interval of 0.02 s. The measurement was repeated three times. One enzyme unit was defined as an absorbance change of 0.01 per minute, and the enzyme activity was expressed in $Unit.g^{-1}FW \cdot min^{-1}$.

Statistical analyses

The data were statistically analysed using Excel and the statistical analysis software, SPSS. The measurements for each sample were repeated three times, and the means were obtained for further analysis. The correlation of the measured results was analysed using the bivariate analysis of correlation (spearman, 2-tailed) of the SPSS program (version 10.01, SPSS Inc., Chicago, IL), and the figures were generated using Microsoft Excel.

RESULTS

Changes in citric acid and malic acid content in developing of 'Xinping' pear fruit

The organic acids with the highest content in pear fruit are citric acid and malic acid, and they have important effects on the quality of the fruit. As shown in Figure 1, the changes in citric acid and malic acid content displayed an overall slowly increasing trend in developing fruit. On day 15 after full bloom, the citric acid content was 0.26 $mg \cdot g^{-1}$ FW, and the malic acid content 1.13 $mg \cdot g^{-1}$ FW in the fruits. Thus, the content of malic acid was higher than citric acid during this period. At day 45 after full bloom, the levels of both acids fell to the lowest levels observed during the entire developmental period, with the citric acid content at 0.02 $mg \cdot g^{-1}$ FW and the malic acid content at 0.94 $mg \cdot g^{-1}$ FW. Malic acid in the fruit rapidly accumulated to the highest level of the entire developmental period, 2.91 $mg \cdot g^{-1}$ FW, at day 90 after full bloom, whereas citric acid accumulated to the highest level of the entire development period, 4.14 $mg \cdot g^{-1}$ FW, at day 135 after full bloom. On the 165th day after full bloom, when the fruits had ripened, there was a rapid decrease in the citric acid content, to 3.01 $mg \cdot g^{-1}$ FW, and

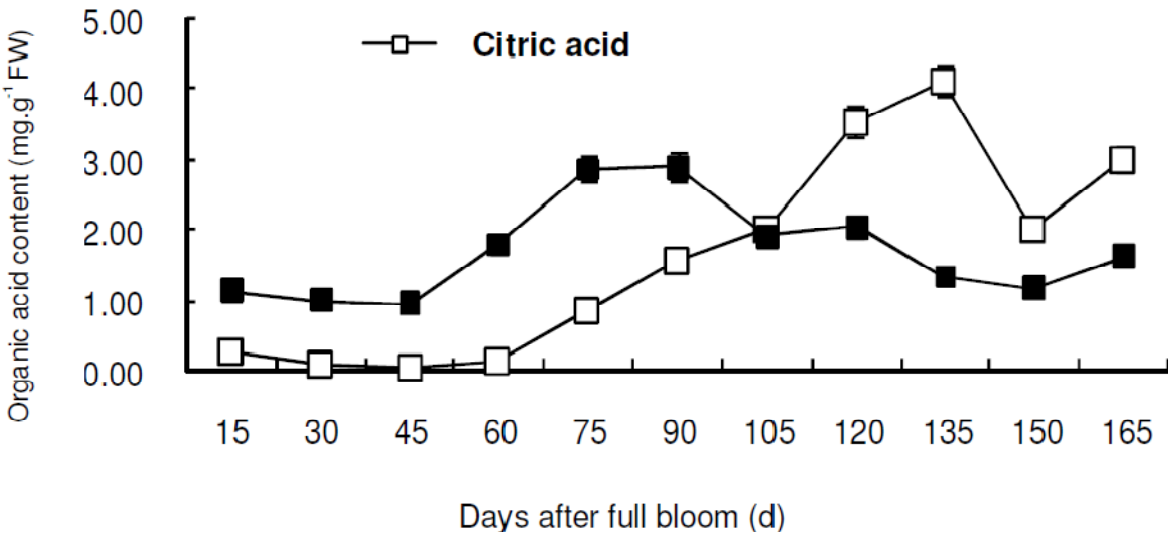

Figure 1. Organic acid content in 'Xinping' pear fruit.

the malic acid content slowly dropped to 1.63 mg·g^{-1} FW; thus, the citric acid content was higher than the malic acid content at this stage.

Changes in the activities of the enzymes involved in citric acid metabolism in developing 'Xinping' pear fruit

Citrate synthase (CS)

The CS enzyme catalyses the condensation of oxaloacetic acid (OAA) and acetyl coenzyme A (Ac-CoA) to form citric acid. Overall, changes in CS activity (Figure 2) showed a slowly increasing trend in developping 'Xinping' pear fruit. On day 15 after full bloom, the CS activity in fruits was 0.85 U·g^{-1} FW·min^{-1}, a relatively low level. At day 45 after full bloom, the CS activity decreased to the lowest level of the entire developmental period, 0.67 U·g^{-1} FW·min^{-1}. By day 135 after full bloom, the CS activity had increased to the highest level of the entire developmental period, 5.45 U·g^{-1} FW·min^{-1}, whereas on day 165 after full bloom (when the fruits were ripe), the CS activity had substantially decreased to 4.01 U·g^{-1} FW·min^{-1}.

Over the fruit developmental period, the correlation coefficient between the CS activity and the citric acid content in 'Xinping' pear fruit was 0.70* (r0.05 = 0.576), suggesting a significant positive correlation between the CS activity and fruit citric acid content.

Aconitase (ACO)

Two aconitase isozymes, mitochondrial ACO (Mit-ACO) and cytosolic ACO (Cyt-ACO), exist in plants. The changes of Cyt-ACO activity, shown in Figure 3, demonstrated an overall slowly decreasing trend in developing 'Xinping' pear fruit. At day 15 after full bloom, the fruit Cyt-ACO activity was 1.42 U·g^{-1} FW·min^{-1}, the highest level of the entire period of fruit development, whereas at day 90 after full bloom, the Cyt-ACO activity had decreased to the lowest level of the entire development period, 0.42 U·g^{-1} FW·min^{-1}. At day 165 after full bloom, the Cyt-ACO activity in the ripe fruits had gradually increased to 0.84 U·g^{-1} FW·min^{-1}.

The analysis of correlation between Cyt-ACO activity and the fruit citric acid content during the fruit development of showed a correlation coefficient of -0.433 (r0.05=0.576), suggesting a negative correlation between the Cyt-ACO activity and the fruit citric acid content during the development of the fruit.

Isocitrate dehydrogenase (IDH)

There are two forms of IDH, NAD-IDH and NADP-IDH. The assay results showed (Figure 4) an overall increasing trend of NAD-IDH activity during the fruit development of the 'Xinping' pear. At day 15 after full bloom, the fruit NAD-IDH activity was 0.18 U·g^{-1} FW·min^{-1}, a relatively low level. At day 60 after full bloom, the NAD-IDH activity had decreased to the lowest level of the entire developmental period, 0.17 U·g^{-1} FW·min^{-1}. At day 90 after full bloom, the NAD-IDH activity had increased to the highest level of the entire development period, 0.65 U·g^{-1} FW·min^{-1}, and by day 165 after full bloom, the NAD-IDH activity had decreased to 0.44 U·g^{-1} FW· min^{-1} in the ripe fruits.

During fruit development of the 'Xinping' pear, the

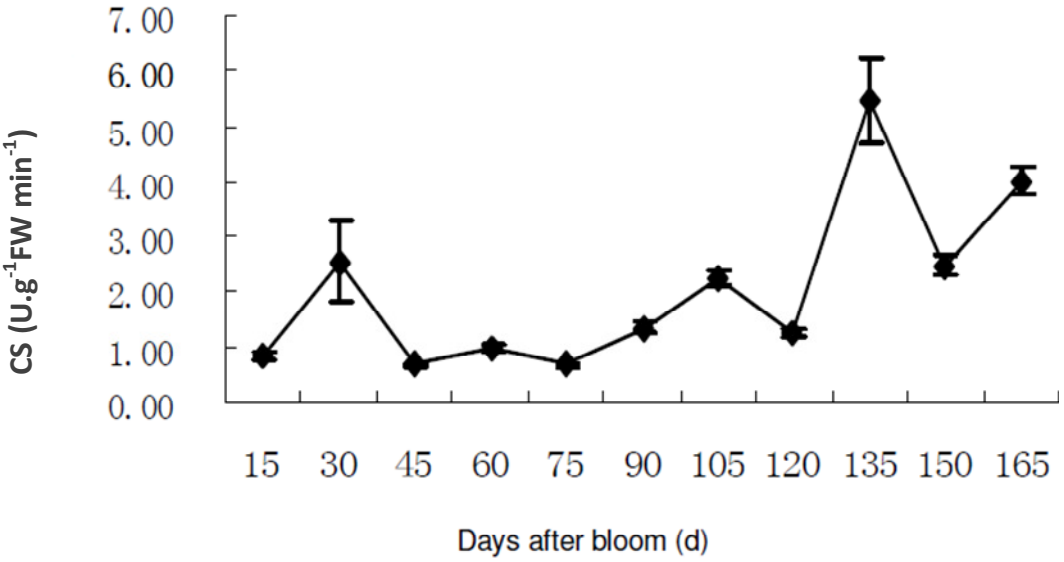

Figure 2. CS activities in developing 'Xinping' pear fruits.

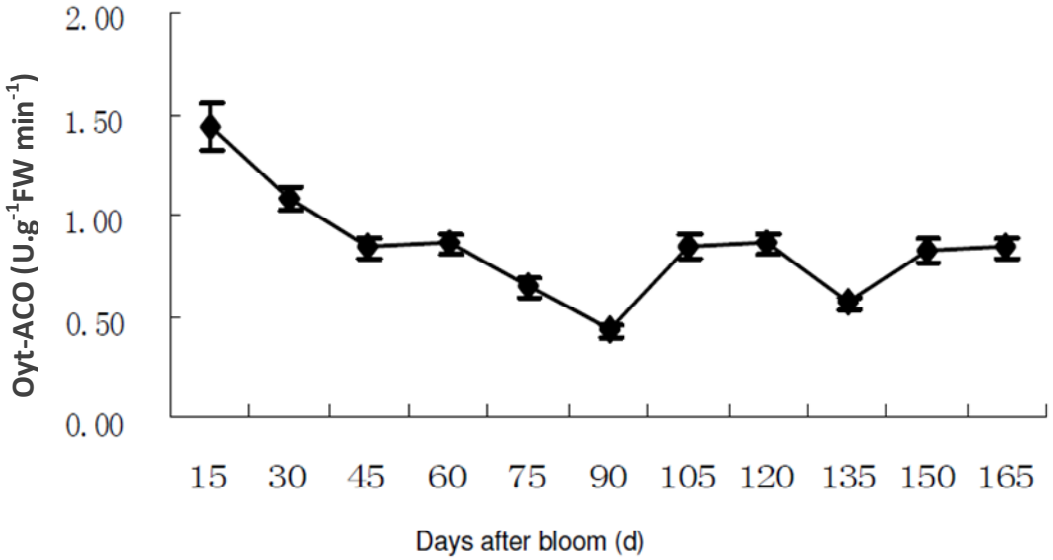

Figure 3. OYT-ACO activities in developing 'Xinping' pear fruits.

correlation coefficient between the NAD-IDH activity and the citric acid content of the fruits was 0.087 (r0.05 = 0.576), suggesting a positive correlation between them. Further analysis revealed that the correlation coefficient between them was -0.544 (r0.05 = 0.754) during the late fruit development (90-165 d), suggesting a negative correlation between the NAD-IDH activity and fruit citric acid content at the late fruit development stage, which indicated that the increase of the NAD-IDH activity at the late fruit development stage resulted in the decrease of the fruit citric acid levels.

Changes in the activities of the enzymes involved in malic acid metabolism in developing 'Xinping' pear fruit

Phosphoenolpyruvate carboxylase (PEPC)

PEPC is the key enzyme for malic acid synthesis in fruits. The results of the PEPC activity assay (Figure 5) showed that PEPC activity had an overall decreasing trend during the fruit development of 'Xinping' pear. On day 15 after full bloom, the fruit PEPC activity was 2.56

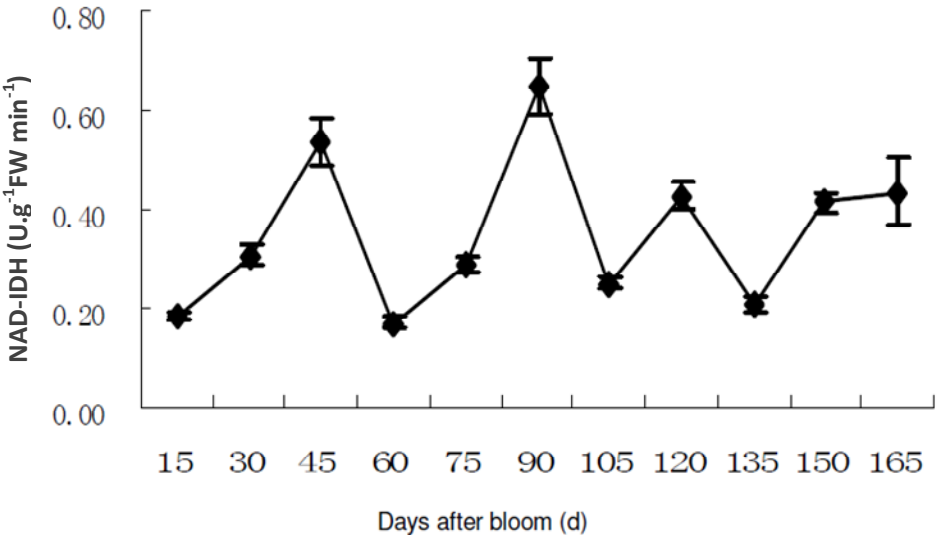

Figure 4. NAD-IDH activities in developing 'Xinping' pear fruits.

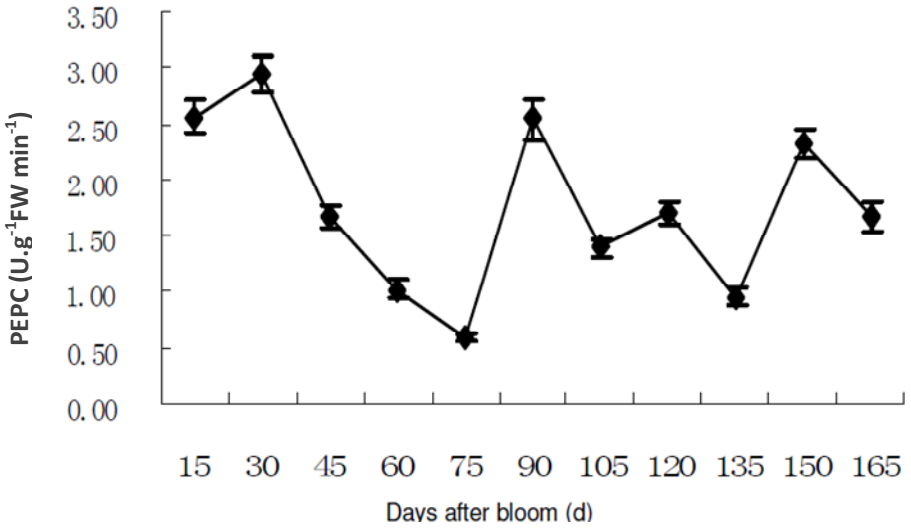

Figure 5. PEPC activities in developing 'Xinping' pear fruits.

$U \cdot g^{-1}$ $FW \cdot min^{-1}$, a relatively high level. On day 30 after full bloom, the PEPC activity increased to the highest level of the entire developmental period (2.95 $U \cdot g^{-1}$ $FW \cdot min^{-1}$) whereas at day 75 after full bloom, the PEPC activity had decreased to the lowest level of the entire developmental period, 0.57 $U \cdot g^{-1}$ $FW \cdot min^{-1}$. At day 90 after full bloom, the PEPC activity had increased substantially to 2.54 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, after which the PEPC activity started to decline and reached 1.67 $U \cdot g^{-1}$ $FW \cdot min^{-1}$ at day 165 after full bloom in the ripe fruits.

Over the entire fruit development, the correlation coefficient between the PEPC activity and the fruit malic acid content was -0.35 (r0.05 = 0.576), suggesting a negative correlation between these during the entire fruit developmental period. Further analysis demonstrated that the correlation coefficient between them at the late fruit development stage was 0.47 (r0.05 = 0.754) (90-165 d), suggesting a positive correlation between the PEPC activity and fruit malic acid content at the late fruit development stage and indicated that the decrease of PEPC activity at the late development stage led to the decrease in the fruit malic acid content.

NAD-malate dehydrogenase (NAD-MDH)

NAD-MDH is an important enzyme involved in malic acid synthesis in the cytosol of plant cells. In general, the

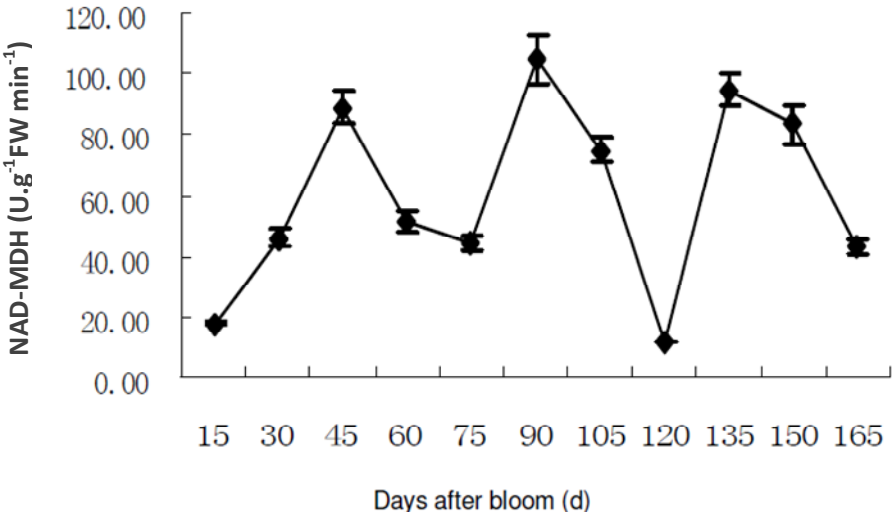

Figure 6. NAD-MDH activities in developing 'Xinping' pear fruits.

results of the NAD-MDH activity assay showed an increasing trend for the NAD-MDH activity over the fruit developmental process of 'Xinping Pear', as shown in Figure 6. On day 15 after the full-bloom stage, the NAD-MDH activity in the fruits was found to be at a relatively low level (17.67 $U \cdot g^{-1}$ $FW \cdot min^{-1}$), which was subsequently found to be increased to the highest level of the entire developmental stage (104.74 $U \cdot g^{-1}$ $FW \cdot min^{-1}$) at day 90. On day 120 after the full-bloom stage, NAD-MDH activity was reduced to the lowest level of the entire developmental stage (11.71 $U \cdot g^{-1}$ $FW \cdot min^{-1}$). The NAD-MDH activity then rapidly increased to 94.90 $U \cdot g^{-1}$ $FW \cdot min^{-1}$ on day 135 after the full-bloom stage and subsequently decreased to 43.46 $U \cdot g^{-1}$ $FW \cdot min^{-1}$ in the ripe fruits on day 165 after the full-bloom stage.

Our correlation analysis results showed a correlation coefficient of 0.044 (r0.05 = 0.576) for the NAD-MDH activity and malic acid content in the fruits over the entire fruit development stage, indicating a positive correlation between the two.

NADP-malic enzyme (NADP-ME)

NADP-ME is a key enzyme that reduces malic acid during the fruit ripening process. As shown in Figure 7, the NADP-ME activity assay results indicated that the NADP-ME activity displayed an overall declining trend during the fruit development of 'Xinping' pear. On day 15 after the full-bloom stage, the NADP-ME activity in the fruits was at a relatively low level (3.70 $U \cdot g^{-1}$ $FW \cdot min^{-1}$), reaching the lowest level of the entire fruit development at 0.43 $U \cdot g^{-1}$ $FW \cdot min^{-1}$ on day 60, whereas the NADP-ME activity increased to the highest level, 28.61 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, by day 120. Subsequently, the NADP-ME activity started to decline and reached a level of 1.19 $U \cdot g^{-1}$ $FW \cdot min^{-1}$ on day 165 after full flowering.

The correlation analysis revealed that the correlation coefficient was 0.286 (r0.05 = 0.576) for the NADP-ME activity and malic acid content in fruits, indicating a positive correlation between the two over the entire fruit development stage. Further analysis showed that this correlation coefficient changed to -0.819 (r0.05 = 0.95) during the late stages of fruit development (135 to 165 days after full flowering), which suggested a negative correlation between the NADP-ME activity and the malic acid content in the fruits during the late stages of fruit development. The result of this negative correlation indicated that the decrease in NADP-ME activity led to the increased fruit malic acid content at the late stages of fruit development.

DISCUSSION

Organic acid content is an important component of fruit quality and affects the flavour and sugar content of the fruit. Different types of fruits have different compositions and contents of organic acids, and the organic acid content usually declines in ripening fruits (Etienne et al., 2002). Malic acid is generally considered the major organic acid in the pear fruit, followed by citric acid. However, the content of citric acid in the fruits of some pear varieties has been found to be equal to or higher than malic acid (Arfaioli and Bosetto, 1993). The present study revealed that the citric acid content was higher than the malic acid content in the ripe fruits of the 'Xinping' pear. During fruit development, CS exhibited high activity, which showed a significant positive correlation with the citric acid content, indicating that the high CS activity in the pear fruit is the major cause for the high citric acid

content. Bruemmer et al. (1977) have obtained similar

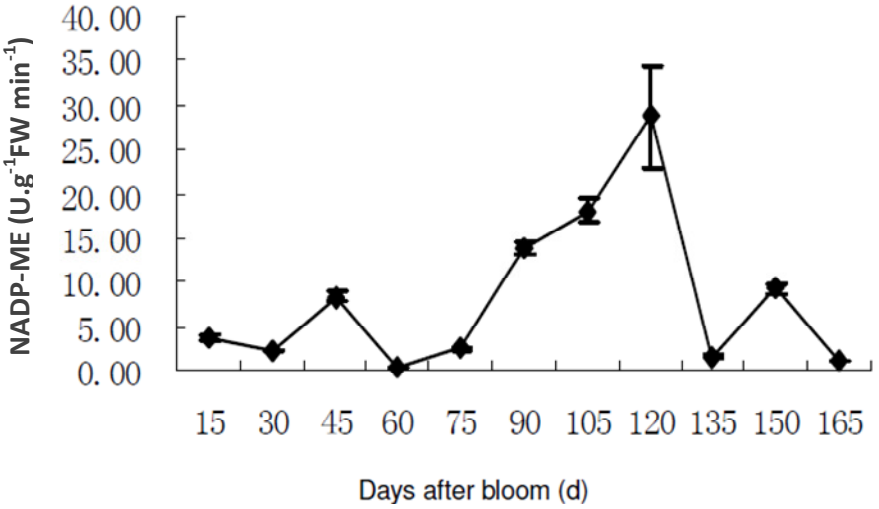

Figure 7. NADP-ME activities in developing 'Xinping' pear fruits.

results in their study with lemon fruits. However, in the study with the fruits of Japanese summer orange (*C. natsudaidai*), Kubo et al. (2002) found that the CS activity was not correlated with citric acid accumulation during the development of the fruit. Therefore, the fruits of different species exhibit significant differences in the activities of the major enzymes involved in the metabolism.

The activity of ACO has been reported to show a certain correlation with the accumulation of citric acid (Sadka et al., 2000b); however, the correlation was found to be insignificant due to the low enzyme activity. A study by Bogin and Wallace (1966) has revealed that the inhibition of ACO activity led to the accumulation of citric acid in citrus fruit. The present study showed a negative correlation between the Cyt-ACO activity and the citric acid content, indicating that the increased citric acid content during pear fruit development was closely related to the decrease in ACO activity.

Organic acid metabolism and its regulation in fruit is a complex physiological process. Both citric acid and malic acid are intermediates of the tricarboxylic acid (TCA) cycle, and their synthesis and degradation are tightly linked to the TCA cycle. In plant cells, PEPC exists only in the cytosol for catalysing the formation of oxaloacetic acid from pyruvic acid and CO_2. The formed oxaloacetic acid is then the substrate for the synthesis of citric acid. Therefore, PEPC is a critical enzyme involved in the dark fixation of CO_2. Haffaker and Wallace (1959) have proposed a synthesis route for citric acid in fruit, in which oxaloacetic acid (OAA) reacts with acetyl coenzyme A (Ac-CoA) to generate citric acid under the catalysis of CS. The present study found a significant positive correlation between the CS activity and citric acid. During the entire fruit developmental process, the CS activity displayed an increasing trend, which was likely to result in the increased accumulation of citric acid in the fruits. Furthermore, IDH showed a weak correlation with citric acid content and, thus, played a minor role in organic acid accumulation in fruits.

Malic acid and citric acid are the major organic acids in pear fruits. During fruit development, the organic acid content slowly elevated and gradually declined during the fruit ripening stage. Correlation analyses demonstrated that the citric acid content had a significant positive correlation with the CS activity and a negative correlation with the ACO activity during the entire fruit developmental process, whereas the citric acid content showed a negative correlation with the NAD-IDH activity at the late stages of fruit development. The malic acid content showed a negative correlation with NAD-MDH during the entire development of the fruit, whereas it exhibited a positive correlation with the PEPC activity at the late stages of fruit development. In addition, the citric acid content displayed a negative correlation with to the NADP-ME activity at the late stages of fruit development. These results indicated that CS, ACO, NAD-IDH, PEPC, MDH and NADP-ME are the principal enzymes that participate in the organic acid accumulation in pear fruits.

The organic acid metabolism in fruits is a complex process that is caused by the integrated regulation of various enzymes and is significantly influenced by the external environment. The organic acid metabolism in pear fruits and its regulatory mechanisms remain to be further investigated at the molecular level.

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