African Journal of Biotechnology Vol. 10(82), pp. 19043-19048, 19 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2258
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effect of metabolic enzyme on organic acids in developing 'Dangshansuli' pear leaf

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Accepted 9 November, 2011

Changes in the content of citric and malic acids and the activities of enzymes involved in the metabolism of these two organic acids, including citrate synthase (CS), cytoplast aconitase (Cyt-ACO), NAD-isocitrate dehydrogenase (NAD-IDH), phosphoenolpyruvate carboxylase (PEPC), NAD-malate dehydrogenase (NAD-MDH) and NADP-malic enzyme (NADP-ME), were examined during the process of leaf development in 'Dangshansuli' pear. The citric acid content exhibited an overall slightly decreasing trend and the malic acid content exhibited an overall appreciably increasing trend in developing leaf. The activities of CS, Cyt-ACO, NAD-IDH, PEPC and NAD-MDH all showed an overall decreasing trend, whereas the activities of NADP-ME indicated an overall increasing trend. The correlation analysis of the change in the organic acid content and related metabolic enzyme activities during the development of the leaf revealed that the activities of CS, Cyt-ACO and NAD-IDH showed a negative correlation with the content of citrc acid, the activities of PEPC exhibited a positive correlation with the content of malic acid, the activities of NAD-MDH and NADP-ME indicated a negative correlation with the content of malic acid.

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

INTRODUCTION

Organic acids have an important role in plant metabolism (Rasmussen and Smith, 1961; Burris, 1953). During plant development, changes in the organic acid content and in the related metabolic enzymes' activities are inseparable (Gallardo et al., 1995; Popova and Pinheiro de Carvalho, 1998; Chen et al., 2001; Iannetta et al., 2004). Citric and malic acids are the major organic acids in pears (Arfaioli and Bosetto, 1993; Hudina and Stampar, 2000), and their levels have an important influence on pear quality. Related research has shown that citrate synthase (CS) (Yamaki, 1990; Wen et al., 2001), aconitase (ACO) (Sadka et al., 2000), and isocitrate dehydrogenase (IDH) (Sadka et al., 2000) are important enzymes affecting the citric acid metabolism of the fruit, whereas phosphoenolpyruvate carboxylase (PEPC) (Wen et al., 2001), malate dehydrogenase (MDH) (Miller et al., 1998), and malic enzyme (ME) (Ruffner et al., 1984) are important enzymes affecting the malic acid metabolism of the fruit. Studies have found that citric and malic acids are also the major organic acids in plant leaves (Dekock and Morrison, 1958; Jayaprakasha and Sakariah, 2002; Jayaprakasha et al., 2003).

Currently, there are a few studies on the role of the organic acid-related metabolic enzymes of leaves in the accumulation of organic acids. Therefore, we used 'Dangshansuli' pear leaves to study the accumulation of citric and malic acids, as well as any changes in the activities of the related enzymes, during leaf development. The objectives were to understand the key metabolic enzymes involved in the accumulation of organic acids in leaves and to explore the mechanism of the accumulation of leaf organic acids in order to inform further studies of fruit organic acid metabolism and its

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Abbreviations: CS, Citrate synthase; **Cyt-ACO,** cytoplast aconitase; **NAD-IDH,** NAD-isocitrate dehydrogenase; **PEPC,** phosphoenolpyruvate carboxylase; **NAD-MDH,** NAD-malate dehydrogenase; **NADP-ME,** NADP-malic enzyme.

Table 1. Organic acid content in developing 'Dangshansuli' pear leaf.

Organic acid	Content ($mg \cdot g^{-1}$) on day after full bloom (days)										
	15	30	45	60	75	90	105	120	135	150	165
Citric acid	1.09	1.03	1.09	1.09	1.02	2.39	1.60	2.18	1.64	1.35	1.05
Malic acid	1.47	0.81	0.67	1.03	1.32	1.80	1.14	1.93	1.52	1.40	1.67

regulatory mechanisms.

MATERIALS AND METHODS

Plant material

The present study was conducted during the leaf-growing season in 2009. The tested materials were from four-year-old 'Dangshansuli' 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. The soil used in the study was sandy loam soil, with a pH of 6.28, a soil organic matter content of 0.54% ($w \cdot w^{-1}$), a total nitrogen content of 0.06%, a phosphorus content of 0.05% and an exchangeable potassium content of 1.82%. The samples were collected from 46 plants of 'Dangshansuli' pear, which were planted with a row spacing of 4 × 3 m, and had spindle-shaped crowns. All of the plants were grown in accordance with local management standards, including pruning, fertilizing and pest control. Samples were collected once every 15 days after the full-bloom stage until the fruits ripened. Ten leaves were randomly taken at each sampling from the upper middle part of the plant canopy, from peripheral shoots with similar growing conditions. The sampling time was 10:00 AM. The leaves were washed, cut into small pieces, and mixed together. Approximately, 10 g of tissue was taken and placed in vials, frozen in liquid nitrogen, and stored at -70°C for later use.

Methods

The organic acid content was measured according to the method published by Nisperos-Carriedo et al. (1992). Samples were accurately measured, and 0.50 g of the pear leaf 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-C18 (4.6 × 250 mm) column. The mobile phase consisted of a 0.2% metaphosphoric acid aqueous solution, the flow rate was 1 $mL \cdot min^{-1}$, 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 and malic acids 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 pear leaf was placed in a prechilled mortar, followed by the addition of 2 mL grinding buffer (0.2 $mol \cdot L^{-1}$ Tris-HCl buffer [pH 8.2], 0.6 $mol \cdot L^{-1}$ sucrose and 10 $mmol \cdot L^{-1}$ 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 min at 4°C. The volume of the resultant supernatant was then fixed at 4 mL using the extraction buffer (0.2 $mol \cdot L^{-1}$ Tris-HCl, pH 8.2, 10 $mmol \cdot L^{-1}$ 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 (Shimadzu, Japan) 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 $Unit \cdot g^{-1}FW \cdot min^{-1}$.

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 (Pearson, 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 and malic acids levels during 'Dangshansuli' pear leaf development

Citric and malic acids are the main organic acids in 'Dangshansuli' pear leaves. As shown in Table 1, changes in the citric and malic acids levels showed that the overall citric acid content of 'Dangshansuli' pear leaves decreased slowly during development, while the overall malic acid content increased slowly. Fifteen days after the full-bloom stage, the leaf citric acid content was 1.09 $mg \cdot g^{-1}$ FW, the malic acid content was 1.47 $mg \cdot g^{-1}$ FW.

Table 2. Metabolic enzyme activity in developing 'Dangshansuli' pear leaf.

Metabolic enzyme	Activity ($U \cdot g^{-1}$ FW.min^{-1}) on day after full bloom (days)										
	15	30	45	60	75	90	105	120	135	150	165
CS	15.24	13.33	18.10	71.11	41.90	11.11	17.78	18.89	13.33	27.62	4.44
Cyt-ACO	8.67	8.67	3.33	2.22	18.33	2.22	1.67	4.44	3.33	10.95	1.90
NAD-IDH	1.67	1.25	0.95	0.56	2.38	0.67	5.00	0.67	0.67	2.38	0.28
PEPC	8.89	4.76	6.67	3.81	10.48	6.67	8.00	6.67	4.76	10.48	6.67
NAD-MDH	217.14	265.71	282.86	239.05	58.89	347.62	327.62	172.38	254.29	42.86	153.33
NADP-ME	12.38	26.67	26.67	3.33	23.81	20.00	3.33	8.33	8.89	26.67	20.00

CS, Citrate synthase; Cyt-ACO, cytoplast aconitase; NAD-IDH, NAD-isocitrate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; NAD-MDH, NAD-malate dehydrogenase; NADP-ME, NADP-malic enzyme.

At this time, the malic acid content was higher than the citric acid content. After this period, the citric acid content decreased slowly until 75 days after the full-bloom stage, when it reached its lowest value for the entire development period, 1.02 $mg \cdot g^{-1}$FW; the malic acid content also decreased slowly until 45 days after the full-bloom stage, when it reached its lowest value for the entire development period, 0.67 $mg \cdot g^{-1}$FW.

Ninety days after the full-bloom stage, the leaf citric acid content was 2.39 $mg \cdot g^{-1}$ FW, which was its maximum value measured during the entire development period; 120 days after the full-bloom stage, the malic acid content was 1.93 $mg \cdot g^{-1}$ FW, which was its maximum value measured during the entire development period. At 165 days after the full-bloom stage, the citric acid content in the leaves decreased from its maximum value to 1.05 $mg \cdot g^{-1}$ FW, while the malic acid content slowly dropped to the value 1.67 $mg \cdot g^{-1}$ FW.

At this time, the citric acid content was still higher than that of malic acid.

Activities of citrate synthase (CS) in developing 'Dangshansuli' pear leaves

CS catalyses the condensation of oxaloacetate (OAA) and acetyl coenzyme A (Ac-CoA) to form citric acid. Overall, CS activity decreased slowly during the development of 'Dangshansuli' pear leaves (Table 2). Fifteen days after the full-bloom stage, the leaf CS activity was 15.24 $U \cdot g^{-1}$ FW·min^{-1}. Sixty days after the full-bloom stage, the CS activity increased to its maximum value for the entire developmental period, 71.11 $U \cdot g^{-1}$ FW·min^{-1}. At 165 days after the full-bloom stage, the CS activity decreased to its minimum value observed during development, 4.44 $U \cdot g^{-1}$ FW·min^{-1}.

Throughout leaf development, the correlation coefficient of CS activity and the citric acid content of 'Dangshansuli' pear leaves was -0.292 ($r_{0.05}$ = 0.576), which shows that CS activity was negatively correlated with the citric acid content during leaf development.

Activities of Aconitase (ACO) in developing 'Dangshansuli' pear leaves

Plants contain two isozymes of ACO, namely, mitochondrial ACO (Mit-ACO) and cytoplasmic ACO (Cyt-ACO). Table 2 shows that during the development of 'Dangshansuli' pear leaves, Cyt-ACO activity generally decreased. Fifteen days after the full-bloom stage, the Cyt-ACO activity in the leaves was 8.67 $U \cdot g^{-1}$ FW·min^{-1}. Seventy-five days after the full-bloom stage, the Cyt-ACO activity was 18.33 $U \cdot g^{-1}$ FW·min^{-1}; which was the highest Cyt-ACO activity measured during the entire period of leaf development. However, 105 days after the full-bloom stage, the Cyt-ACO activity decreased to its minimum observed value, 1.67 $U \cdot g^{-1}$ FW·min^{-1}; 165 days after the full-bloom stage, the Cyt-ACO activity slowly increased to 1.90 $U \cdot g^{-1}$ FW·min^{-1}.

During this development of the 'Dangshansuli' pear leaves, the correlation analysis of Cyt-ACO activity and citric acid content revealed a correlation coefficient of -0.384 ($r_{0.05}$ = 0.576), which indicates that Cyt-ACO activity was negatively correlated with leaf citric acid content during development.

Activities of isocitrate dehydrogenase (IDH) in developing 'Dangshansuli' pear leaves

IDH exists in two forms, namely, NAD-IDH and NADP-IDH. The results (Table 2) show that during 'Dangshansuli' pear leaf development, the overall NAD-IDH activity demonstrated a decreasing trend. Fifteen days after the full-bloom stage, the NAD-IDH activity of the leaves was 1.67 $U \cdot g^{-1}$ FW·min^{-1}. At 105 days after the full-bloom stage, the NAD-IDH activity increased to 5.0 $U \cdot g^{-1}$ FW·min^{-1}, which was the highest level observed during the entire developmental period. However, at 165 days after the full-bloom stage, the NAD-IDH activity decreased to its minimum value, 0.28 $U \cdot g^{-1}$ FW·min^{-1}.

Over the course of the development of 'Dangshansuli' pear leaves, the correlation coefficient of NAD-IDH

activity and leaf citric acid content was -0.063 ($r_{0.05}$ = 0.576), which suggests that they were negatively correlated during leaf development.

Activities of phosphoenolpyruvate carboxylase (PEPC) in developing 'Dangshansuli' pear leaves

PEPC is a key enzyme in the synthesis of malic acid. The PEPC activities measured in this experiment (Table 2) showed that the overall PEPC activity of the 'Dangshansuli' pear leaves decreased during development. Fifteen days after the full-bloom stage, the PEPC activity of the leaves was 8.89 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, but at 60 days after the full-bloom stage, the PEPC activity decreased to its minimum value, 3.81 $U \cdot g^{-1}$ $FW \cdot min^{-1}$. At 75 days after the full-bloom stage, the PEPC activity increased rapidly to its maximum value observed during development, 10.48 $U \cdot g^{-1}$ $FW \cdot min^{-1}$. After this, the PEPC activity decreased through 165 days after the full-bloom stage to 6.67 $U \cdot g^{-1}$ $FW \cdot min^{-1}$.

Over the entire developmental period of 'Dangshansuli' pear leaves, the correlation coefficient of PEPC activity and leaf malic acid content was 0.193 ($r_{0.05}$ = 0.576), which indicates that they were positively correlated throughout the entire developmental period.

Activities of NAD-malate dehydrogenase (NAD-MDH) in developing 'Dangshansuli' pear leaves

NAD-MDH is an important enzyme in malic acid synthesis and is found in the cytoplasm. In Table 2, the results of NAD-MDH activity measurements showed that NAD-MDH activity appears to decrease throughout the developmental period. Fifteen days after the full-bloom stage, the NAD-MDH activity of the leaves was 217.14 $U \cdot g^{-1}$ $FW \cdot min^{-1}$. At ninety days after the full-bloom stage, the NAD-MDH activity increased to its maximum value, 347.62 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, of the entire developmental period, but at 150 days after the full-bloom stage, the NAD-MDH activity decreased to its minimum value observed during development, 42.86 $U \cdot g^{-1}$ $FW \cdot min^{-1}$. When fruit reached maturity at 165 days after the full-bloom stage, the NAD-MDH activity increased to 153.33 $U \cdot g^{-1}$ $FW \cdot min^{-1}$.

Correlation analysis revealed that throughout the development period of 'Dangshansuli' pear leaves, the correlation coefficient of NAD-MDH activity and leaf malic acid content was -0.216 ($r_{0.05}$ = 0.576), which indicates that they were negatively correlated throughout the entire developmental period.

Activities of NADP-malic enzyme (NADP-ME) in developing 'Dangshansuli' pear leaves

NADP-ME is a key enzyme in the metabolism of malic acid. In Table 2, NADP-ME activity measurements show that the overall NADP-ME activity in developing 'Dangshansuli' pear leaves appears to have an increasing trend. Fifteen days after the full-bloom stage, the NADP-ME activity in the leaves was 12.38 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, which was relatively low. At 30 days after the full-bloom stage, the NADP-ME activity rapidly increased to its maximum observed value, 26.67 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, but at 60 days after the full-bloom stage, the NADP-ME activity decreased to its minimum observed value, 3.33 $U \cdot g^{-1} FW \cdot min^{-1}$. After that the NADP-ME activity began to increase until the fruit was mature at 165 days after the full-bloom stage, at which point the NADP-ME activity was 20.0 $U \cdot g^{-1}$ $FW \cdot min^{-1}$.

Correlation analysis showed that the correlation coefficient of NADP-ME activity and leaf malic acid content was -0.244 ($r_{0.05}$ = 0.576), which indicates that they were negatively correlated throughout the entire developmental period.

DISCUSSION

Citric and malic acids are the most important organic acids in the leaves of many types of fruit trees, and their levels vary among different species. In the mature leaves of most fruit trees, the malic acid content is higher than the citric acid content. The malic acid and citric acid contents in pineapple leaves are highest at 9 AM. At this time, the malic acid content is greater than the citric acid content (Peng et al., 1998). In different varieties of loquat leaves, the malic acid content is in the range of 0.777-0.226 $mg \cdot g^{-1}FW$, and the citric acid content is in the range of 0.228-0.026 $mg \cdot g^{-1}FW$; the malic acid contents in all of these varieties are higher than the citric acid contents (Chen et al., 2006; Chen et al., 2004). Zhang et al. (2002) found that malic acid was the major organic acid in persimmon leaves. However, the citric acid content was also higher than the malic acid content in the leaves of individual trees. In plum leaves, the citric acid content was 1.24 $mg \cdot g^{-1}$ FW, while the malic acid content was 0.64 $mg \cdot g^{-1}$ FW; therefore, the citric acid content was higher than the malic acid content in this species (Pan et al., 2008). This study found that the malic acid content was higher than the citric acid content in 'Dangshansuli' pear leaves in the early and late stages of development.

Haffaker and Wallace (1959) proposed that within fruit, citric acid biosynthesis is catalysed by CS, which combines oxaloacetate (OAA) with acetyl coenzyme A (Ac-CoA) to synthesise citric acid. In experiments on lemon fruit, Bruemmer et al. (1977) found that the change in CS activity was the main cause of the increase in citric acid content. In this experiment, CS activity appeared to decrease during the development of the 'Dangshansuli' pear leaves, and it was negatively correlated with citric acid content. It can be concluded that in developing 'Dangshansuli' pear leaves, the CS activity is not the main factor causing the decrease in citric acid content.

Kubo et al. (2002) found using Japanese Valencia oranges that CS activity is not associated with the accumulation of citric acid. Therefore, the roles of CS in different organs of different types of fruit trees are very diverse.

It has been reported that ACO activity is correlated with the accumulation of citric acid (Sadka et al., 2000a), but due to the low enzymatic activity, the correlation is not significant. Bogin and Wallace's (1966) study suggested that the inhibition of ACO activity in citrus fruit causes the accumulation of citric acid. The present study found that Cyt-ACO activity was negatively correlated with citric acid content, indicating that in the development of 'Dangshansuli' pear leaves, the decrease in citric acid content is closely related to the decline in ACO activity.

NAD-IDH mainly exists in the mitochondria and participates in the TCA cycle, catalysing the decarboxylation of isocitrate to generate α-ketoglutarate (2-OG); so changes in the activity of this enzyme affect the citric acid content. In this experiment, the NAD-IDH activity of the leaves was slightly negatively correlated with citric acid content. The decrease in NAD-IDH activity during 'Dangshansuli' pear leaf development had little impact on citric acid content.

A study by Ruffner et al. (1984) in grapes found that NAD-MDH always functioned together with PEPC; the oxaloacetate generated by PEPC catalysis was converted to malic acid by the action of MDH. Iannetta et al. (2004) indicated that as strawberry fruits ripened, the malic acid content increased, and the MDH activity also significantly increased. NADP-ME may be related to the conversion of malic acid. Liu et al. (2007) found that in rice plants, through the metabolism of malic acid, NADP-ME was involved in the adaptation to salt stress and osmotic stress. The correlation analysis in this study showed that over the entire developmental period of the leaves, the malic acid content was positively correlated with PEPC activity, which was negatively correlated with the activities of NAD-MDH and NADP-ME. The analysis showed that the malic acid content increased and was not related to the changes in PEPC, NAD-MDH and NADP-ME activities in developing 'Dangshansuli' pear leaves.

Conclusion

Organic acid metabolism in leaves is a complex process that is under the control of different enzymes; in addition, it is influenced by the external environment. The metabolism and control mechanisms of organic acids in pear fruit and leaf need to be further studied at the molecular level.

ACKNOWLEDGEMENTS

This work was supported by the Earmarked Fund for Modern Agro-industry Technology Research System (nycytx-29) and Key Technologies and Program of Jiangsu (BE2010324)

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