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RESEARCH ARTICLE

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Effect of microenvironments and exogenous substance application on 5′-nucleotidase activities in apple peel

Abstract The present experiment was conducted to examine the effect of microenvironments and exogenous substance application on 5′-nucleotidase activity in apple peel tissue. By enclosing apple fruits in bags, treating them with exogenous active oxygen species and regulative agents or placing them under controlled conditions at different fruit temperatures or relative humidity, the 5′-nucleotidase activities were compared with the corresponding controls. The results indicated that, a considerable effect of the microenvironments was found on 5′-nucleotidase activities in fruit peel tissue. The highest enzymatic activity appeared in fruits on the southwest exposure of canopy, regardless of bagged or non-bagged fruits, significantly higher than those from any other exposures. Fruits with bags had a significantly higher 5′-nucleotidase activity than the exposed ones. A variation in enzymatic activities was observed in fruits enclosed with different types of bags, which were supposed to alter the microenvironments around them. Within a certain range, gradual or fluctuating rise of fruit temperatures could favor the increase of 5′-nucleotidase activities as a result of heat adaptation, whereas the activity would be inhibited if the temperature-rising period was too short or temperature differential was too large. No matter what temperatures fruits were subjected to, high relative humidity was favorable for stimulating the 5′-nucleotidase activities, which might partly explain why fruit sunburn would not happen in humid climates. Treatments with four kinds of exogenous active oxygen species could reduce the 5′-nucleotidase activities significantly but spraying with $CaCl_2$ was able to enhance 5′-nucleotidase activities by 55.39%, reaching a 5% significant level.

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1 Introduction

Apple fruits are frequently stressed during growth and development by environmental factors, such as high temperatures, low humidity and excessive solar radiation. Bagging could create a particular microenvironment for fruits, and in some situations, the intensity of stress like temperature was aggravated (Simpson et al., 1988; Pan and Xin, 1995). As the adaptation of plant to stress adversities depends largely upon the property of the membrane (Su, 2000), it is therefore theoretically important to understand how environmental stresses affect membrane function. 5′-nucleotidase (EC 3.1.3.5) has been classified as an enzyme belonging to hydrolyric category and mainly bound on the plasma membrane, which has been widely used for studies on cold tolerance or resistance of some agricultural crops. It has been recognized as one of the characteristic enzymes (Wang et al., 1994; Chen et al., 1997). Unfortunately, the literature on 5′-nucleotidase's effect on fruit crops is extremely limited so far and no report is actually available on the response of this enzyme to oxidative stresses caused by high temperature, low humidity as well as exogenous formulations. The present experiment aims at examining the effect of different microenvironments and formulations on 5′-nucleotidase activity to provide a sound basis for further pursuing the mechanism and effective preventions.

2 Materials and methods

2.1 Location and materials

The experiment was conducted at Beizhang orchard, Baoding City, Hebei province as well as in the key-discipline laboratory of pomology, Ministry of Agriculture of the People's Republic of China, situated at Agricultural University of Hebei. Lab experiments and field trials were mainly carried out in 2002 and 2003, respectively. Twelve-year-old apple trees (*Malus domestica* Borkh. cv. Fuji) were used which were grown on crab apple rootstocks (*M. micromalus* Mill.). The planting space was 3 m × 5 m. Trees grew and bore well and the orchard was managed normally. Trees of similar sizes and consistent vigor were chosen as samples, which also had good light reception and even load (about 45 kg per tree).

2.2 Design

2.2.1 Comparison of enzymatic activity among different exposures

On three sample trees, the four exposures of SE, NE, SW and NW were divided on the canopy and on each of them, three uniformly-growing fruit were selected and bagged with double-layer bags in late May and an additional three were left bare as the control. A single tree served as a plot and random block arrangement was adopted with three replicates. In sunny days around mid July, the temperatures of fruit including bagged and the control were measured with JM222 digital temperature probe at 1.5 h intervals, beginning from 8:00 in the morning. At 16:00 in the following day, both bagged and control fruits were harvested. In the laboratory, fruit peel on the exposed side (containing epidermal layer and adjacent flesh tissue, about 0.5–1 mm in thickness) was removed and then put into extra-cold refrigerator (−72°C). As far as determination of significant difference among treatments was concerned, Duncan's new multiple range test was applied for statistical analysis (same as below).

2.2.2 Effect of different types of bags on enzymatic activity

A single tree served as a plot and trees were block-randomly arranged with three replicates. Three fruits on the SW exposure of each tree were bagged by chance in late May, and the treatments included: (1) Double-layer paper bags (DP); (2) Single-layer black paper bags (SP); (3) White plastic film bags (WF); and (4) Control (CK).

The size of the above bags mentioned was 20 cm × 14.5 cm. The sample fruits were collected in sunny days after mid July for assay of enzymatic activity.

2.2.3 Effect of fluctuating temperatures on enzymatic activity

In mid August, uniformly detached fruits were covered with clear plastic wrap and put into a water incubator. Three treatments were applied: (1) gradual temperature rising (T1): fruit temperatures increased from 25°C to 48°C via four stops (25°C → 30°C → 40°C → 48°C); (2) direct temperature rising (T2): fruit temperatures increased from 25°C to 48°C via two stops (25°C→48°C); (3) fluctuating temperature rising (T3): fruit temperatures increased from 25°C to 48°C via four stops (25°C → 45°C → 25°C → 48°C). Treatments of both T1 and T3 lasted for 24 h. During treatment, fruit stayed at that temperature for 4 h whenever fruit temperatures reached stop temperatures of either 25°C, 30°C, 40°C or 45°C, and as soon as fruit temperature reached 48°C, they were allowed to stay at that temperature for 20 min. The surplus time was distributed evenly among the process of heating. After treatment, fruits were peeled and put into a refrigerator. A complete random-block design was adopted with three replicates (three fruits serving as a plot).

2.2.4 Effect of relative humidity (RH) on enzymatic activity at different temperatures

In mid August, uniform bagged fruit were put into a growth chamber (Model: DL-302A). Temperatures were set at 35°C, 40°C or 48°C, respectively, and RH was set at 60% or 80% for 2 h. During treatments, fruit surface temperatures were monitored and time was counted as soon as fruit temperature reached the designed value. Fruits were peeled immediately after treatment before putting into a refrigerator. A complete random-block design was also adopted with three replicates (three fruits serving as a plot).

2.2.5 Effect of different exogenous active oxygen species on enzymatic activity

In early September, uniform bagged fruits were treated with the following four formulations, which enabled the generation of active oxygen species (AOS), with water soaking as the control: (1) superoxide anion (Source: 50 mmol/L sodium hydrosulfite); (2) singlet oxygen (Source: 10 mmol/L H_2O_2 + 1 mmol/L NaClO); (3) hydrogen peroxide (Source: 50 mmol/L 30% H_2O_2); (4) hydroxyl radical (Source: 20 mmol/L EDTA + 500 mmol/L H_2O_2 + 10 mmol/L Fe_2SO_4). Fruits stayed at room temperature for 4 h after treatment and then were peeled before being refrigerated. A complete random-block design was adopted in the same way.

2.2.6 Effect of exogenous formulations on enzymatic activity

Fifteen trees were chosen, random-block designed was adopted with three replicates and single tree served as a plot. Uniform fruits on the SW exposure of the tree canopy were bagged in late May and on September 20, the fruit bags were removed and fruits were sprayed with the following four formulations: (1) $CaCl_2$ (20 mmol/L); (2) DDC (0.01 mol/L); (3) Sodium benzoate (SBN) (0.1 mol/L); (4) Ascorbic Acid (AsA) (0.1 mol/L); and (5) Water (Control). Fruits were sprayed in a clear morning and picked up at 16:00 of the following day. Three fruits were harvested from each plot. In the laboratory, fruits were peeled and refrigerated.

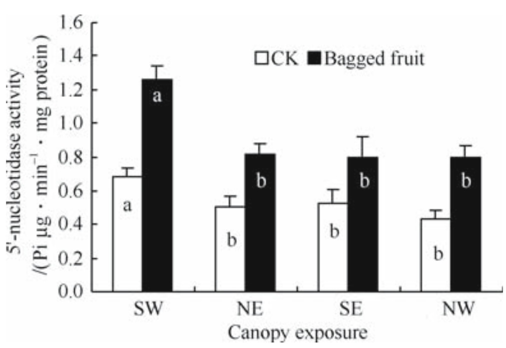
2.3 Assay of 5′-nucleotidase activity

The method modified by Pan Jie and Li Meiru was followed (Pan, et al., 1992; Li, et al., 1996). 0.3 g peel tissue was ground in 3 mL buffer solution (50 mmol/L Hepes-KOH (pH 7.5) + 25 mmol/L KCl + 5 mmol/L $MgCl_2$). The syrup was filtered through a layer of gauze and the liquid was centrifuged at 15 000 r/min for 20 min. The supernatant liquid was prepared for assay. The enzyme and reaction solutions were added into a test tube. The reaction solution contained 30 mmol/L Hepes-KOH (pH7.5), 1 mmol/L $MgCl_2$, 5 mmol/L 5′-AMP and 5 mmol/L $NaMoO_4$ while the same solution without 5′-AMP was used as the blank control. After the reaction was executed at 30°C for 15 min, 300 μL 20% trichloroacetic acid was added to terminate the reaction. After centrifugation, the supernatant was collected for determining the contents of inorganic phosphorus by Molybdenum blue method and protein content was assayed with Coomassie brilliant blue method. The unit of 5′-nucleotidase activity was expressed as Pi $\mu g \cdot min^{-1} \cdot mg^{-1}$protein.

3 Results

3.1 Comparison of enzymatic activity among different exposures of canopy

Great variations existed in the microenvironments where fruits grew among different tree exposures because of the considerable daily changes of sunlight reception and other ecological conditions. In terms of comparison of 5′-nucleotidase activity on different exposures, it was shown that the activity of fruits on the SW exposure was significantly higher than that on all other exposures with insignificant difference among them, which was probably associated with a higher fruit surface temperature present on the SW exposure that had been proven before (Zhang et al., 2002; Zhang et al., 2003). In addition, the bagged fruits on all exposures had a higher 5′-nucleotidase activity than that of the corresponding controls (Fig. 1), probably resulting from higher fruit temperatures due to bagging as well (Just take SW exposure as an example herein, and the similar trend follows on other exposures, Fig. 2). It was assumed that the acclimation to heat induced the increase of 5′-nucleotidase activity (Fig. 3).



The difference between bagged and control on all exposures reached 1% significant level

Fig. 1 Effect of different canopy exposures on 5′-nucleotodase activity in fruit peel

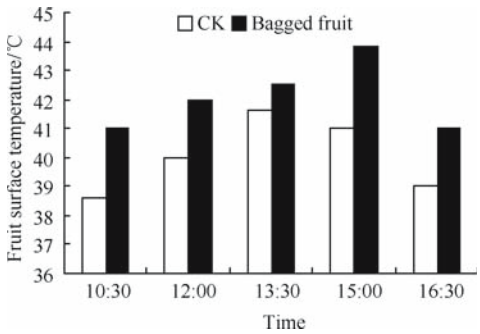


Fig. 2 Comparison of fruit surface temperatures between bagged and exposed fruits

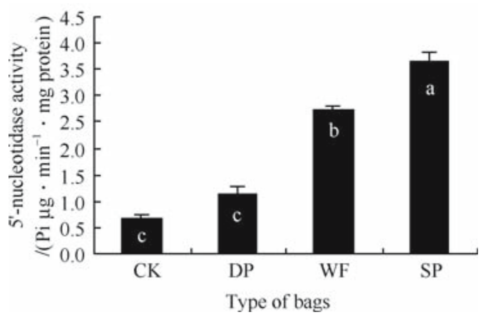


Fig. 3 Effect of different types of bags on 5′-nucleotodase activity

3.2 Effect of different types of bags on enzymatic activity

Different effects of various types of bags were found on 5′-nucleotidase activity in peel tissue and a higher level of activity existed in the bagged fruits, which was likely to be related to a higher fruit temperature caused by bagging. Based on the measurement on daily maximum fruit surface temperatures in a clear day, fruits covered with different types of bags are ranked in a descending order: Black paper bags (44.2°C) > White plastic film bags (44°C) > Double-layer paper bags (43.8°C) > Control (41.6°C), which was positively coincident to the level of enzymatic activity. Among different types of bags, the fruits enclosed with black paper bags had the highest activity, increasing by 436.12% in comparison to the control, achieving very significant level. Similarly, fruits with plastic bags were significantly higher than the control but there was no statistical difference between double-paper bags and the control.

3.3 Effect of fluctuating temperatures on enzymatic activity

Fluctuating temperatures could affect 5′-nucleotidase activity substantially (Fig. 4). Gradual rising of temperature (25°C → 30°C → 40°C → 48°C) had the highest activity because it was favorable for the acclimation of fruit to high temperatures, and fluctuating rising of temperature (25°C→45°C→25°C→ 48°C) could also induce 5′-nucleotidase activity to increase significantly than the control even though fruit temperatures underwent an up-and-down change. However, direct rising of temperature (25°C→48°C) resulted in an insignificant increase of enzyme level partly because fruits were hardly adapted to a higher temperature within a short period of time even though fruit temperatures increased eventually from low to high. Therefore, it was suggested that within a certain range, gradual rising of temperature favored the acclimation of fruit to high temperatures, whereas the contrary was true if the temperature rising process proceeded too quickly or the temperature differential was too large.

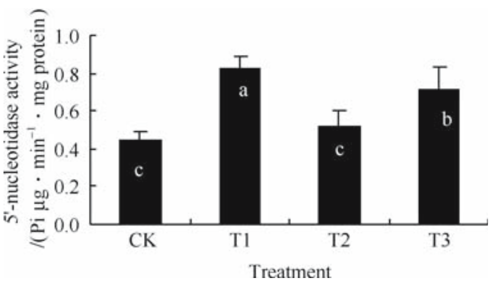
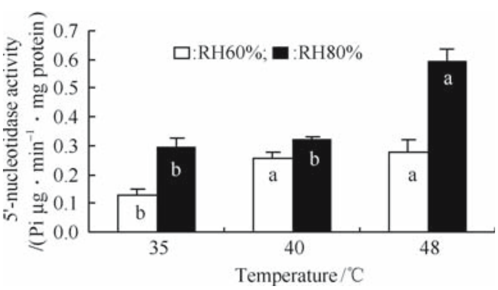


Fig. 4 Effect of temperature-rising modes on 5′-nucleotidase activity

3.4 Effect of RH on enzymatic activity

Certain effects of RH were observed on 5′-nucleotidase activity (Fig. 5). Apparently, the 5′-nucleotidase activity was higher in high humidity than that in low humidity, regardless of levels of temperature treatments (Insignificant difference at 40°C). At the same humidity, the changes of 5′-nucleotidase activity varied with fruit temperatures. For instance, no obvious change was found in 5′-nucleotidase activity at 85% RH when fruit temperatures retained 35°C and 40°C, but when fruit temperature held at 48°C, 5′-nucleotidase activity increased abruptly. However, 5′-nucleotidase activity had a greater increase at 60% RH when fruit temperatures were kept at 35°C and 40°C. Nevertheless, the increasing scope was smaller between 40°C and 48°C, indicating that the enzyme was insensitive both to high temperature at lower RH and to low temperature at higher RH.



At 35°C and 48°C, there is a significant difference between RH 60% and RH 80% but no significant difference at 40°C

Fig. 5 Effect of different humidity on 5′-nucleotidase activity

3.5 Effect of different exogenous active oxygen species on enzymatic activity

Different exogenous active oxygen species could aggravate the oxidative stress in fruit. The results indicated that a great influence of different AOS existed on 5′-nucleotidase activity (Fig. 6). In the present experiment, four kinds of AOS decreased the 5′-nucleotidase activity consistently with insignificant difference present among them, suggesting that AOS could accentuate the lipid peroxidation to injury of membrane integrity, resulting in a lower 5′-nucleotidase activity.

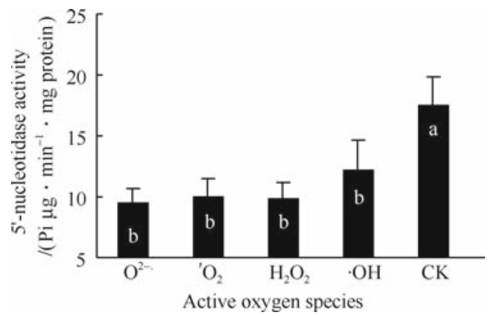


Fig. 6 Effect of different exogenous active oxygen species on 5′-nucleotidase activity

3.6 Effect of different exogenous formulations on enzymatic activity

5′-nucleotidase activity varied with different exogenous formulations (Fig. 7). Actually from the experiments, it can be seen that the formulations used in experiments could serve as different functions. As is known, DDC is a kind of inhibitor of Superoxide dismutase (SOD); SBN is a scavenger for free radicals; AsA is a typical antioxidant; and $CaCl_2$ (Ca^{2+}) is an important second-messenger with other extensive functions. The results indicated that only $CaCl_2$ increased 5′-nucleotidase activity by 55.39%, reaching a significant level in comparison to the control. Moreover, SBN and AsA had a trend to raise 5′-nucleotidase activity, increasing by 23.79% and 31.60%, respectively, whereas DDC had a trend to reduce 5′-nucleotidase activity, which were likely responsible for their particular physiological function. In conclusion, spraying with exogenous $CaCl_2$ could raise 5′-nucleotidase activity, which is favorable for strengthening and improving membrane integrity. Our conclusion was basically consistent to some relevant reports (Su, 2000).

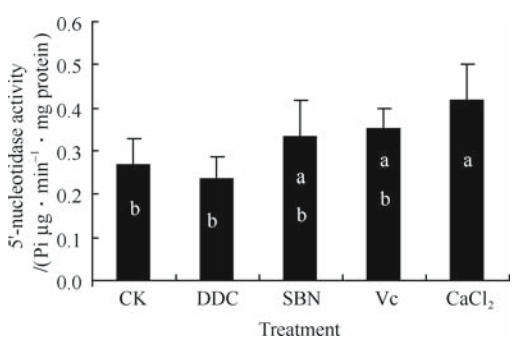


Fig. 7 Effect of exogenous agents on 5′-nucleotidase activity

4 Discussion

4.1 Fruit heat resistance influenced by microenvironments

5′-nucleotidase is mainly bound on plasma membrane and associated with energy metabolism, nutrient absorption and transportation as well as synthesis of nuclear acids, RNA degradation and ATP metabolism (Chen et al., 1996; Chen et al., 1997). Therefore, it can clearly be seen that 5′-nucleotidase activity is related closely to membrane function. It has been documented that the adaptation of plant to stressed adversities depends mainly upon the property of the membrane (Su, 2000), thus 5′-nucleotidase activity could be used as a sensitive indicator to reflect the status of membrane function. It was observed in our experiment that, the changes of fruit microenvironments, as a consequence of bagging, temperature and RH alternations, etc., could result in a variation in 5′-nucleotidase activity of peel tissue accordingly. Moreover, the pattern of changes seemed to be associated with the extent of stress by the environments. Within a certain range, 5′-nucleotidase activity increased with a rise of temperature and RH. Certainly, it is worthwhile to distinguish whether the above-mentioned changes are a consequence of fruit to heat acclimation or an immediate response to heat stress. Based on the principles of resistance physiology, heat adaptation must happen within a range of temperatures and enzymatic activity would be definitely inhibited when temperatures exceed a certain threshold scope. Therefore, it is necessary to study the exact threshold values of heat adaptation with respect to 5′-nucleotidase activity, so that the optimal conditions of acclimation could be proposed. Practically, fruit surface temperatures as well as RH and light intensity within the particular microenvironment must be modified greatly as bagging culture is recently being popularized in China, which will surely affect fruit development and resistance. For this reason, it is of great theoretical and practical significance to examine the relationship between the microenvironment and 5′-nucleotidase activity in fruit peel, in relation to fruit resistance to various adversities.

4.2 Significance of 5′-nucleotidase activity adjustment

Now that 5′-nucleotidase activity is directly related to membrane function and further resistance to adversities (Eastwell and Stumpf, 1982; Yang and Endo, 1994), an increase of 5′-nucleotidase activity by means of application of proper measures before environmental stress, in a sense, should be beneficial to improve fruit resistance to heat stress. The preliminary results indicated that 5′-nucleotidase activity could be raised by altering the microenvironments where fruits grow, and intervening in the process of fruit acclimation to adversities, such as high temperature, low RH, etc. As soon as fruits acquire a certain oriented acclimation, fruit could possibly obtain more resistance to the stress when it comes. Of course, application of exogenous formulations may also have the potential to adjust 5′-nucleotidase activity to some extent apart from changes of ecological conditions. This will definitely open a new prospect in the study on oxidative stress of apple fruit.

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