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ANALYSIS OF PHYSIOLOGICAL INDEXES AND AROMA-RELATED GENES OF THICK-SKINNED MELON (*CUCUMIS MELO* L. CV. HETAU) UNDER SALT STRESS

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Abstract

Comprehensive performance and aroma synthase in melon seedling under salt stress could directly affects the aromas content of its fruit. In the present research, when the height of the *Cucumis melo* L. cv Hetau was 10~15 cm, the seedlings were treated using Hoagland with 0, 50, 100, 150 and 200 mM NaCl. After three days of NaCl stress, the tender leaves of *C. melo* L. cv Hetau were cut for the extraction of total RNA, semi-quantitative PCR was used to analyze the lipoxygenase (LOX) and alcohol dehydrogenase 3 (ADH_3) gene expression in *C. melo* L. cv Hetau under NaCl stress, and after 40 days, samples were taken for the determination of chlorophyll content, root activity based on α-naphthylamine content, nitrate reductase activities (NR), LOX activities and the alcohol dehydrogenase (ADH) activities in *C. melo* L. cv Hetau. The results showed that the relative expression of *LOX* and ADH_3 genes increased with NaCl increase, and reached the highest level under 100 mM NaCl, the relative expression of the genes decreased under 150-200 mM NaCl. The chlorophyll content, root activity based on α-naphthylamine content and ADH activities in *C. melo* L. cv Hetau rose first, and then decreased with NaCl increase. The chlorophyll contents, root activities based on α-naphthylamine content and LOX activities reached the highest value under 50 mM NaCl, and ADH and NR activities reached the highest value at 100 mM NaCl. These results indicate that higher NaCl can obviously impact the physiological reaction of *C. melo* L. cv Hetau, and the impact is more obvious with the increase of NaCl. The study indicated that the moderate salt could adjust the aroma of muskmelon to the best.

Key words: Physiological indexes, Aroma synthesis enzyme, *C. melo* L. cv Hetau, NaCl stress.

Introduction

Plants are subjected to numerous types of biotic and abiotic stresses during growth. Salinity is one of the major abiotic stresses in agriculture worldwide (Munns, 2002), excess salt in the soil adversely affects plant growth, development and productivity. Salt stress mainly damage to plants through osmotic stress, ion toxicity, and also affects multiple physiological activities of plants (Xu *et al*., 2020). To respond to external stresses, the expression of a variety of genes is induced in plants, leading to physiological and metabolic changes that increase stress tolerance (Zhu, 2002). Melon is an important horticultural plant with rich nutrition and high economic value. In 2020, the world's melon planting area was 1.07 million hectares, with a yield of 28 million tons, and China's melon production accounted for more than 49%, creating an export value of approximately US$0.13 billion, making it the second largest economic crop in the *Cucurbitaceae* family (www.fao.org/faostat/en/). Therefore, planting high-quality melon is the basis for obtaining high benefits and achieving extensive processing of melon. Melon is an economically important crop that is extensively cultivated in semiarid regions (Wei *et al*., 2013), farmers in these regions tend to use irrigation water that mainly contains chloride and sodium ions (Botía *et al*., 2005), this makes salt an important problem in the process of planting melon.

The studies on the effect of salt stress on muskmelons mainly focus on the comparison of gene identification and expression levels between different muskmelons (Shin *et al*., 2019), autotoxic stress (Zhang *et al*., 2020), salt tolerance (Sarabi *et al*., 2017) and the role of medical care (Akrami & Arzani, 2019). Sarabi's research found that Iranian melon seedlings showed a significant increase in the activity of SOD and other indicators with increasing concentrations at low to moderate concentrations (0, 20, 40, 60 and 80 mM NaCl) (Sarabi *et al*., 2017). There have been studies focusing on the content of Na^+/K^+ in transgenic *Arabidopsis thaliana* with K^+ channel gene of melon (Yuan *et al*., 2019). At present, there are some related studies on the related components and genes of muskmelon aroma (Jin *et al*., 2016; Huang *et al*., 2018). However, there are few studies on the changes of physiological indicators and the analysis of aroma gene expression levels in *C. melo* L. cv Hetau under NaCl stress.

The objective of this study was to determine the effects of NaCl stress on aroma quality of *C. melo* L. cv Hetau. We measured photosynthetic pigments, root activity, NR activities and enzyme activities related to aroma synthesis (LOX and ADH) in C. melo L. cv Hetau seedlings under different NaCl concentrations. In addition, the expression activity levels of *LOX* and ADH_3 genes in *C. melo* L. cv Hetau under different concentrations of NaCl stress by semi-quantitative PCR.

Materials and Methods

Plant materials and experimental design: Seeds of *C. melo* L. cv Hetau were used as materials. For the planting and treatment of materials, the screened *C. melo* L. cv Hetau fully matured and seeds of uniform size were cultured in a constant-temperature incubator at 20°C, germinated and transplanted into a nutrition bowl based on a nutrient: soil ratio of 1:3 under natural light at 15~25°C.

When the height of the seedlings reached 10~15 cm, they were treated with Hoagland solution with 0, 50, 100, 150 and 200 mM NaCl solution once every 10 days, 100 mL of NaCl solution was administered three times during 40 days in order to ensure the continuous stress on the seedlings. After three days of stress, the tender leaves of the cantaloupe were cut for the extraction of total RNA to analyze the genes expressions, and after 40 days, samples were taken for the determination of various physiological indexes, with five replications (Wang *et al*., 2019; Li *et al*., 2019).

Measurements of photosynthetic pigments: Photosynthetic pigments were extracted from the leaves collected by cutting 0.2 g of leaves into pieces in a mortar with a mixture of 95% (v/v) alcohol, $CaCO_3$ and SiO_2. Then, the leaf pieces were soaked for 5 min in darkness. The absorbances of the solution were then measured at 440, 645 and 663 nm on a UV-7504 single-beam UV-VIS spectrophotometer (Shanghai, China) for the chlorophyll content. The photosynthetic pigment concentration was calculated according to the following formulas (Kong *et al*., 2020):

$$Chla = 12.71 \times OD_{663} - 2.59 \times OD_{646} \quad (1)$$

$$Chlb = 22.88 \times OD_{645} - 4.67 \times OD_{663} \quad (2)$$

$$Chla + b = 20.29 \times OD_{645} + 8.04 \times OD_{663} \quad (3)$$

$$Ccar = 4.7 \times OD_{440} - 0.27 \times Chla + b \quad (4)$$

Chla: the concentration of chlorophyll a
Chlb: the concentration of chlorophyll b
Chla+b: total chlorophyll concentration
Ccar: carotenoid concentration

OD_{440}, OD_{645} and OD_{663} are the absorption values of chlorophyll solution at wavelengths of 440 nm, 645 nm and 663 nm, respectively.

Root activity: The absorbance was measured at a wavelength of 510 nm, and the concentration of α-naphthylamine was determined according to the standard curve (Guo & Yu, 2012). In this method, the content of α-naphthylamine under different salt concentrations was calculated using the standard curve and the following formula:

All alpha-naphthylamine in solution=initial value of experiment-(value of blank control+ amount of alpha-naphthylamine at end).

$$\text{Oxidation strength of alpha – naphthylam ine } [\mu g/(g.h)] = \frac{25 \times X}{W \times t}$$

χ: the concentration of oxidized α-naphthylamine (μg/mL)
25: the volume of reduced alpha-naphthylamine in the sample (mL)
W: the sample fresh weight (g)
T: the time of oxidation

Nitrate reductase activities: The **Nitrate reductase** (NR) activity was measured and a standard curve was made according to the methods of Cai (2014). 1g of four leaves cut into 1cm cubes was weighed in 10 ml of analysis buffer (100 mM KNO_3, sterile water, 100 mM phosphate buffer, pH 7.5). Before culturing for 20 minutes, move the leaves and solution to the syringe and repeatedly suck until the leaves sink into the solution. The nitrite were determined colorimetrically at 520 nm by adding 1% P-aminobenzenesulfonic acid and 0.2% α- naphthylamine to 2 ml solution and incubating 30℃ for 20 min. The activity of nitrate reductase was calculated according to the following formula (Zhang *et al*., 2021):

$$\text{NR activity } [\mu NaNO_2/(g.h)] = \frac{\text{Determination } (\mu NaNO2) - \text{Contrast } (\mu NaNO2)}{\text{Fresh leaf weight (g) x Reaction time}(h)} \text{x Total volume of reaction solution } (mL)$$

Aroma synthesis related-enzyme activities

LOX Enzyme: The preparation method of substrate was carried out according to the method of Tang & Luo (2012). 0.5 mL of Tween was dissolved in 10 mL of 0.05 M boric acid buffer with pH 9.0, and 0.5 mL of linoleic acid was added. The components were then mixed evenly into an emulsion, and 1.3 mL of 1 M NaOH was added until the solution was clear, after which 90 mL of 0.05 M boric acid buffer at pH 9.0 was added. The pH was adjusted to 7.0 with HCl, and the volume was adjusted to 200 mL. Before use, the solution was diluted 40 times to make a solution of linoleic acid with a concentration of 2.57 mM.

0.5 g leaves were extracted with enzyme solution, and 5 mL of 1 M phosphate buffer (pH 7.5) was added, after which the leaves were ground in an ice bath. After mixing, the tubes were left for 30 min and then centrifuged at 8000 r/min at 4°C for 10 min. The supernatant was the enzyme extract. Then, 0.3 mL of linoleic acid and 60 μL of enzyme extract were added to 9.5 mL of 0.05 M sodium acetate buffer with pH 5.6, and the solution was poured into a quartzite with an optical path of 1 cm. The absorbance was measured at 234 nm and was read once every 15 s with the substrate as a control. The activity of lipoxygenase was calculated according to the following formula.

$$\text{Liposygena se activity } [U/(g.\text{min})] = \frac{\Delta A_{340} \text{x} V_T}{0.001 \text{x} Vs \text{x} t}$$

ΔA340: change of absorption value in reaction time
W: fresh weight of the leaves/g
V_T: total volume of extracted enzyme solution/mL
Vs: volume of enzyme solution taken during determination/mL

ADH enzyme: The extraction method of the crude enzyme solution was determined by referring to the method of Tang (2004). Two grams of *C. melo* L. cv Hetau seedling leaves was weighed in a mortar, 5 mL of precooled enzyme extraction buffer was added, and the mixture was ground into a homogenate in an ice bath. After homogenization, the mixture was filtered with 4 layers of gauze, the filtrate was centrifuged at 8 000 r/min at 4°C for 20 min, and the supernatant was used for enzyme activity determination.

Then, 2.85 mL of enzyme activity buffer and 100 µL of crude enzyme extract were combined and mixed well, and 30 µL of 95% ethanol was added to start the reaction. The absorbance change was measured at 340 nm, and an increase in the ΔA340 by 0.001 per minute was taken as an enzyme activity unit (U). The activity of alcohol dehydrogenase was calculated according to the following formula:

$$\text{Alcohol dehydrogenase activity } [U/(g.\text{min})] = \frac{\Delta A_{340} \text{x} V_T}{0.001 \text{x} V_S \text{x}\ t}$$

ΔA340: change in absorption value within the reaction time
W: leaf fresh weight/g
V_T: total volume of extracted enzyme solution/mL
Vs: volume of enzyme solution taken during the measurement/ mL

Semi-quantitative PCR analysis of lipoxygenase and alcohol dehydrogenase: The expression of the target gene was determined by adjusting the number of PCR cycles and the concentration of cDNA to make the reference brightness of the electrophoresis map consistent. The specific primers *Cm28S rRNA*-F (5'-GCCGACCC TGATCTTCTGTGA-3') and *Cm28S rRNA*-R (5'-TACCCAAGTCAGACGAACGATT-3') were designed based on the conserved sequence of the *28S rRNA* gene in *C. melo* L. available in the NCBI database. The specific primers *CmLOX*-F_1 (5'-ACCAGACTACAACTACTTCG AG-3'), *CmLOX*-R_1 (5'-ACTGGGACAGTTATTCTGG ATG-3'), *CmADH*$_3$-F_1 (5'-ACTGTCGTTCACGATG TCAGTG-3') and *CmADH*$_3$-R_1 (5'-ATGCCAATTATA CGTGAAGCAC-3') was designed based on the *CmLOX* and *CmADH*$_3$ gene sequence.

The total RNA isolated from *C. melo* L. cv Hetau subjected to 0, 50, 150, 200 mM NaCl for 72 h was used to synthesize the first-strand cDNA by the M-MLV reverse transcriptase kit (Promega). The *CmLOX*, *CmADH*$_3$ gene and the control (*28S rRNA*) were used to detect their expression changes by semi-quantitative RT-PCR. The amplification conditions were as follows, 95°C for 5 min; 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min (26 cycles for *28S rRNA*, 28 cycles for *CmLOX* and *CmADH*$_3$), and a final extension at 72°C for 10 min. For the target gene, the PCRs were carried out in three biological repeats.

Statistical analysis

All treatments were performed in triplicate. The data determined in triplicate were visualized with Origin 8.6. Individual difference among means was determined by Duncan's test. Before the ANOVA, the data were checked for both homogeneity and normality of variance, and were log-transformed to correct deviations from these assumptions when needed. Statistical analyse was conducted with the SPSS 19.0 software.

Results and Discussion

Changes in photosynthetic pigments in *C. melo* L. cv Hetau under NaCl stress: Photosynthesis is the basic life activity of crops, it includes a series of complex physiological and biochemical processes. It is also an important metabolic process in dry matter formation of crop and is the main source of material and energy for plant life activities (Wang *et al*., 2020). In order to conduct photosynthesis, photosynthetic pigment absorbs and transmit light energy to PS I and PSII. The chlorophyll is one of the most important pigments during photosynthesis, and its contents of plant leaves reflected the level of photosynthesis to a certain extent. When the chlorophyll is reduced, plant photosynthesis will be weakened, resulting in reduction of plant growth or even death (Kong *et al*., 2020). Chlorophyll is a key component of the thylakoid membrane, the chlorophyll content of leaves is directly related to the photo-contractual process of plants. Photosynthesis is especially sensitive to external environmental conditions (salt, water and temperature, etc.) (Xiong *et al*., 2018). Salt stress affected the chlorophyll content and the absorption and conversion of light energy, thereby inhibiting photosynthesis in plants (Carter & Cheeseman, 1993). We found the chlorophyll contents in *C. melo* L. cv Hetau increased, and reached a maximum under 50 mM NaCl, when the NaCl was more than 50 mM, the photosynthetic pigments decreased with NaCl increase. So low-concentration NaCl could promote the synthesis of chlorophyll a, chlorophyll b and total chlorophyll, the change trend of carotenoid was similar to the chlorophyll, while high NaCl concentration reduce the synthesis of the photosynthetic pigments (Fig. 1). There are two main possible reasons for this phenomenon, one may be attributed to impaired chlorophyll biosynthesis, and the other may be attributed to accelerated pigments degradation (Ashraf & Harris, 2013). It has been argued, however, that increased levels of toxic Na^+-induced inhibition of chlorophyll biosynthesis was more severe.

Changes in the root activity of *C. melo* L. cv Hetau under NaCl stress: The root of the plant is an important organ for absorbing nutrients and water, and is the first part of the plant to feel the salt damage, is also the first site response to the salt stress, therefore, the growth and development of the root under salt stress are crucial to the salt tolerance of plants. Evaluating the root performance under salt stress in each melon variety is of great significance for screening and cultivating of the salt-tolerant varieties (Cramerr *et al*., 1986; Mao *et al*., 2020). Studies on effects of environmental conditions on roots activity of melon are extensive, such as effects of diseases and insect attack on roots activity (Huang *et al*., 2017), and the analyses on transcriptome and metabonomics of melon root was conducted under copper stress (Hu *et al*., 2020). However, there are few reports on root vigor of muskmelon under salt stress. Our experiments suggested that the root activity of *C. melo* L. cv Hetau increased under 0 and 50 mM NaCl, and reached a maximum at 50 mM NaCl, the root activity decreased with more NaCl. The result demonstrated that low NaCl could improve the root vigor of *C. melo* L. cv Hetau (Fig. 2). Relevant studies have also been reported on *Apocynum venetum* (Ning *et al*., 2010) which is an annual herbaceous halophyte. The root activity of *Apocynum venetum* under 100~200 mM NaCl was significantly higher than that of the control (without NaCl). This means there exists a difference in the salinity threshold in root activities between halophyte and non-halophyte, it also means that effect of salt stress on the root vigor of *C. melo* L. cv Hetau could be more severe than *Apocynum venetum's* indicating that *C. melo* L. cv Hetau may be a non-halophyte with certain salt tolerance.

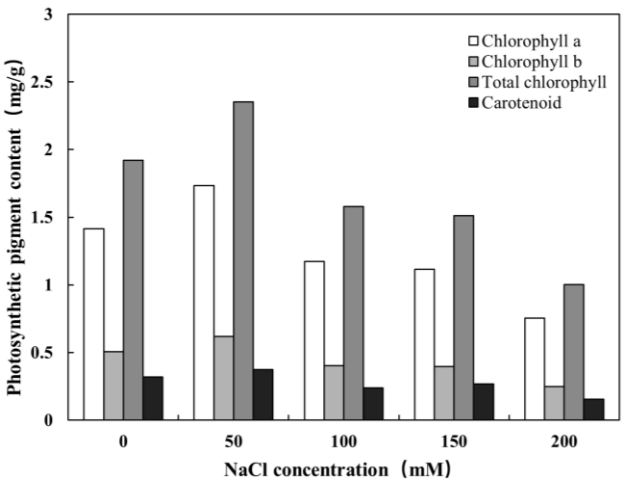

Fig. 1. Photosynthetic pigments in *C. melo* L. cv Hetau under 0~200 mM NaCl.
Note: The error is within the range of 0.000348-0.003208.

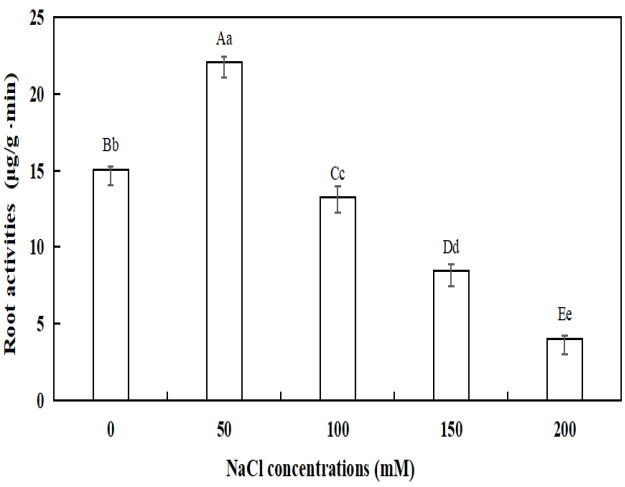

Fig. 2. Root activity based on α-naphthylamine content in C. melo L. cv Hetau at 0~200 mM NaCl.
The capital letters means significant differences at $p \leq 0.01$, and the lower case letter means significant differences at $p \leq 0.05$.

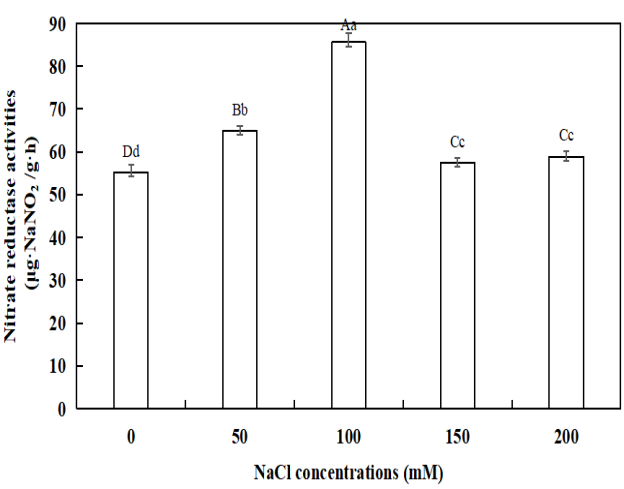

Fig. 3. NR activity in *C. melo* L. cv Hetau at 0~200 mM NaCl. The capital letters means significant differences at $p \leq 0.01$, and the lower case letter means significant differences at $p \leq 0.05$.

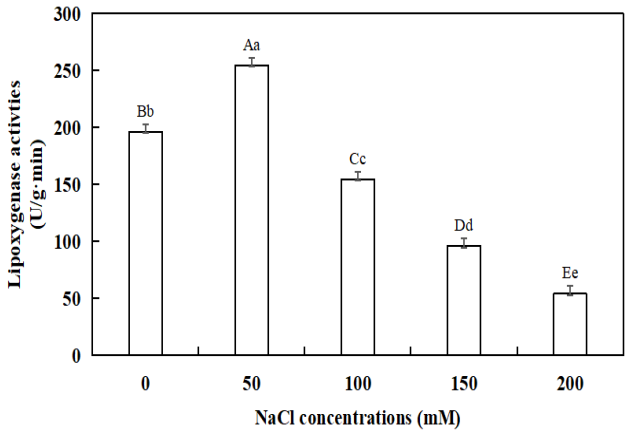

Fig. 4. LOX activities in *C. melo* L. cv Hetau at 0~200 mM NaCl. The capital letters means significant differences at $p \leq 0.01$, and the lower case letter means significant differences at $p \leq 0.05$.

Changes in the NR activity of *C. melo* L. cv Hetau seedlings under NaCl stress: NR plays a centeral role in plant nitrogen assimilation and metabolism (Chamizo-Ampudia *et al*., 2017), which significantly affects the growth and development of plants (Kyaing *et al*., 2011), particularly, high NR activity in leaves is also beneficial to dry matter accumulation and growth of the seedling (Shi *et al*., 2009). The NR activity of *C. melo* L. cv Hetau seedlings increased to a certain extent with NaCl increase, which indicated that moderate NaCl could improve the ability of muskmelon seedlings to utilize nitrogen (Fig. 3), which is roughly consistent with the previous result (Liu & Zhao, 2005). The reason may be low Na^+ has inducing and promoting effects on NR activity, while high Na^+ has toxic effects on the structure and the activity of NR. Reda *et al*., (2011) studied the effect of 200 mM NaCl on NR activity in cucumber roots after salt treatment for 15 min, a 50% stimulation of NR activity was observed. The above studies showed that NR activity was affected by salt stress.

The activity and gene expression of aroma synthase in *C. melo* L. cv Hetau after NaCl stress: LOX is the key enzyme for the synthesis of plant aroma substances, and studies have also shown that plant can regulate LOX activity to reduce H_2O_2 under salt stress, and to enhance its salt tolerance. In this work, we studied LOX activities in *C. melo* L. cv Hetau seedlings under NaCl stress, and the results showed that LOX activities were significantly increased at first and then decreased with NaCl increase, and the maximum value of the LOX activities appeared under 50 mM NaCl. When the NaCl was more than 100 mM, the LOX activity decreased slowly (Fig. 4), similar results were obtained during studies on LOX activity in muskmelon (Wang *et al*., 2019). The expression level of *LOX* gene were similar to the changes of LOX activities with the NaCl increase, the relative expression of *LOX* increased between 0 mM and 100 mM NaCl, and reached the highest level under 100 mM NaCl. Then, the relative expression of *LOX* decreased at more NaCl (Fig. 5). Studies have shown that NaCl stress induced high oxidative stress, increasing lipoxygenase activity to high levels (Vahid *et al*., 2010). Our results agree with the previous reports in other plants (Zhang *et al*., 2013), It also showed that LOX enzyme could be involved in the response of the Hetau melon to salt stress. The related mechanism still needs further study.

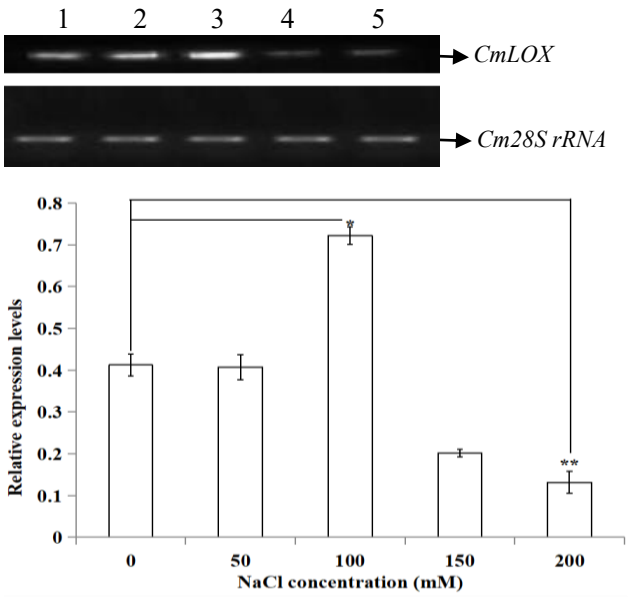

Fig. 5. *LOX* gene expression levels under NaCl stress.

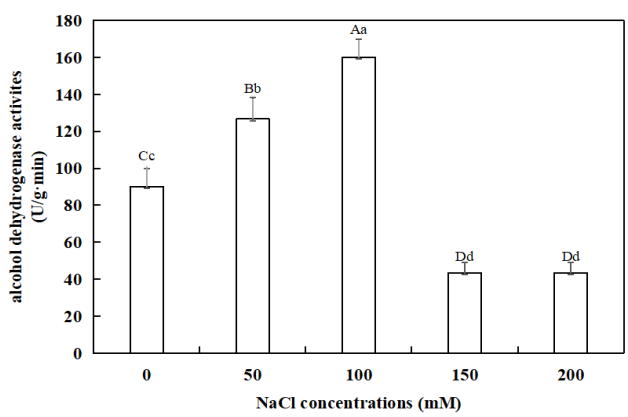

Fig. 6. ADH activity in *C. melo* L. cv Hetau at 0~200 mM NaCl. The capital letters means significant differences at $p \leq 0.01$, and the lower case letter means significant differences at $p \leq 0.05$.

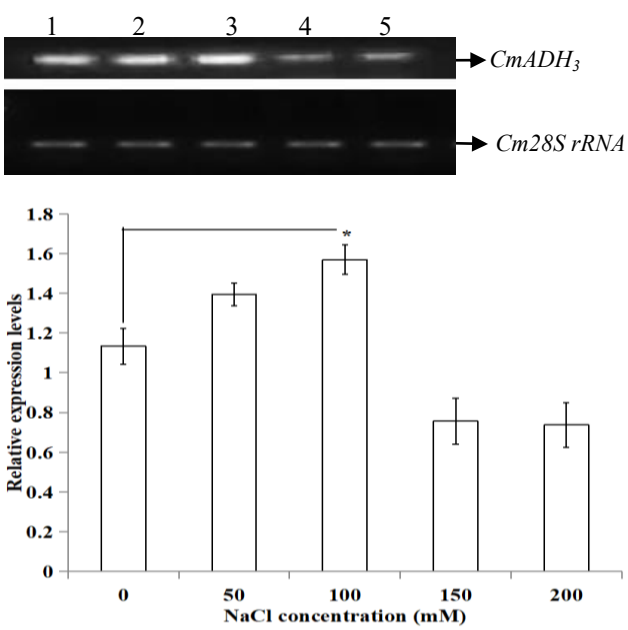

Fig. 7. ADH_3 gene expression levels under NaCl stress.

In addition to rich in nutrients and excellent source of human bioactive substances, muskmelon is also provided with excellent flavor quality (Petkova & Antova, 2015). Many studies have shown that acetate plays a great role in the formation of aroma. The formation of aromatic substances with fatty acids as precursors requires four major enzyme systems, namely, the alcohol dehydrogenase (ADH), the lipoxygenase (LOX), the lipid hydroperoxide lyase (HPL), and the isomerase. ADH is the key enzyme of the LOX pathway; therefore, in order to understand the effects of salt tolerance on aroma quality in melon, it is necessary to study the changes in the activities and gene expression of ADH under salt stress. The ADH activities in *C. melo* L. cv Hetau seedlings first increased and then decreased with NaCl increasing. The ADH activities increased at 0~100 mM NaCl indicating that moderate NaCl could improve its activity. When the NaCl was more than 100 mM, the activities of ADH decreased continuously (Fig. 6). The ADH_3 expression first increased and then decreased under NaCl stress. Under 0~100 mM NaCl, the expression of ADH_3 increased constantly, and reached the highest level under 100 mM NaCl. Then, the expression of ADH_3 decreased in turn under 150 and 200 mM NaCl (Fig. 7). In a word, the change trend on ADH activities and expression of ADH_3 is consistent under sodium salt stress, and the values reached the maximum at 100 mM. The *ADH* gene in Banana is moderately up-regulated under salt stress (Jia *et al*., 2014). Liang *et al*., (2012) reported that NaCl had effect on the expression of ADH_3. Sha's study (2015) showed that NaCl had significant inhibitory effect on ADH_2 in maize seedling roots under 50 mM and 150~250 mM NaCl, while ADH_2 expression was significantly stimulated at 100 mM NaCl. The above research results were not exactly the same as the ADH changed trend of our experiment. The possible reason is that the materials are different. This means NaCl stress has an impact on ADH enzyme. The results of this study can provide important guideline for obtaining melon with better aroma quality.

Acknowledges

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