Physiological Advantages of Grafted Watermelon (*Citrullus lanatus*) Seedlings under Low-temperature Storage in Darkness

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Additional index words. *Citrullus lanatus*, *Cucurbita moschata*, grafting, antioxidant enzyme, photosynthetic rate, seedling quality

Abstract. Low-temperature storage in darkness is usually used for preserving seedlings for a short period. To investigate whether grafted watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai] seedlings are superior to non-grafted ones under low-temperature storage in darkness and to study their physiological differences during storage, watermelon ('Zaojia 84-24') scions were grafted to pumpkin (*Cucurbita moschata* Duch. 'Zhuangshi') rootstocks. Carbohydrate levels; chlorophyll and malondialdehyde contents; the activities of superoxide dismutase, catalase, and peroxidase; and photochemical efficiency were assayed during 6 days of storage at 15 °C in darkness. After that, seedlings were transplanted into an artificial climate chamber. The net photosynthetic rate and stomatal conductance (g_s) were measured on the first and third days after transplanting. The results showed that the grafted watermelon seedlings had more soluble sugar and chlorophyll contents, higher activities of antioxidant enzymes, and less malondialdehyde content than the non-grafted ones after 6 days of storage. In addition, low-temperature storage in darkness damaged the photosystem II of non-grafted watermelon seedlings more than that of grafted ones. After transplanting, grafted seedlings had a higher net photosynthetic rate. The results suggest that grafted watermelon seedlings were more suitable for the low-temperature storage in darkness than the non-grafted ones.

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai] seedlings are the main product of the seedling companies in China. With the increases in the cultivation area of watermelon (2.2 million ha in China and 3.8 million ha in the world; FAOSTAT, 2009) and the demand for watermelon seedlings (≈33 billion per year in China), seedling storage is essential for meeting market demands. The most common method of preserving seedlings for a short-term period is low-temperature storage in darkness (Kaczperski and Armitage, 1992). Although low-temperature storage preserves seedling vigor and inhibits overgrowth, it reduces seedling quality to some extent. The interruption of photosynthesis and the low-temperature stress were regarded to be the important factors that affect the physiological changes of the seedlings during storage (Ning et al., 2006a).

Grafting is universally used for watermelon production to resist root diseases (Beltrán et al., 2008) and to increase the tolerance to salinity (Goreta et al., 2008) and low-temperature stress (Liu et al., 2003). Justus and Kubota (2010) reported that grafted muskmelon (*Cucumis melon* L.) seedlings had better storability under low-temperature storage with 12 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density and suggested that the probable reason was the result of their higher chilling tolerance compared with the non-grafted ones. Thus, we expected that higher tolerance of grafted watermelon seedlings against low temperature could contribute to their storability. In this study, we compared the physiological responses of grafted and non-grafted watermelon seedlings during low-temperature storage under darkness and their photosynthetic recovery after transplanting to investigate whether grafted watermelon seedlings are superior to non-grafted ones in storability.

Materials and Methods

Plant materials and grafting method. Watermelon [*C. lanatus* 'Zaojia 84-24'] seeds and pumpkin (*Cucurbita moschata* Duch. 'Zhuangshi') rootstock seeds were provided by a seedling company (Shanghai Yuanyi Seedling Co., Ltd., Shanghai, China) and sown in 72-cell plastic plug trays (50 cm³

per cell). For subsequent grafting, rootstock seeds were sown 5 d before scion seeds. The composition of substrate was 40% peat, 40% vermiculite, and 20% perlite. When scion seedlings had two expanded cotyledons and the rootstock seedlings grew one small true leaf, grafting was performed with the hole-insertion method described by Hassell et al. (2008). After grafting, grafted seedlings were maintained in a healing chamber at 27 ± 2 °C temperature, 95% ± 5% relative humidity, and 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation (*PAR*). The healing process was complete after 6 d, and grafted seedlings were transferred into a greenhouse with 26 ± 2/20 ± 2 °C (day/night) temperature and 60% to 80% relative humidity. Non-grafted watermelon seedlings with the same seedling age were used as the control and nursed in the same greenhouse as the grafted ones. The water content of the substrate for both grafted and non-grafted seedlings was kept at 75% to 85% of the maximum water-holding capacity with a nutrient solution (18N–7.9P–14.9K–1.5Mg) daily. The electrical conductivity of the nutrient solution was 1.0 ms·cm⁻¹.

Storage and transplant conditions. Fifteen d after grafting, seedlings with two fully expanded true leaves and one small true leaf were transferred into growth chambers (Model HP-400 GC-C; Wuhan Ruihua Instrument & Equipment Co., Ltd., Wuhan, China) under dark conditions, 15 °C air temperature, and 85% to 95% relative humidity (Holcomb, 1994). Each treatment was repeated in three growth chambers, and each chamber held a grafted and a non-grafted seedling tray. Trays were relocated within and among chambers every 2 d to minimize environmental differences. Before storage, the water content of the substrate in each tray was adjusted to 75% of the maximum water-holding capacity using the weighting method (Chen et al., 2002). Seedlings were not watered during storage.

After 6 d of storage, seedlings were transplanted into the 24-cm diameter plastic pots filled with the same medium as in the seedling nursery and were transferred into an artificial climate chamber at 28/22 °C (day/night) temperature, 70% to 80% relative humidity, 14-h photoperiod, and 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ *PAR*. Plants were watered daily with half-strength Hoagland's solution.

Quantification of carbohydrates and chlorophyll. Fresh leaves (0.5 g) were boiled in 10 mL water at 95 °C for 20 min. After centrifugation at 12,000 g_n for 10 min, the residue was extracted three times using the same method. The water extract was used for the determination of soluble sugar levels with the anthrone-H₂SO₄ reagent (McCready et al., 1950). The insoluble fraction was hydrolyzed with 2 mL of 9.2 M HClO₄ for the determination of starch levels according to McCready et al. (1950).

Chlorophyll was extracted from 0.2 g fresh leaves by 10 mL of 80% acetone for 48 h in darkness. The extract solution was measured using a spectrophotometer (Model Helios α ; Thermo Electron Corp., Waltham, MA) at 663.2 and 646.8 nm. The chlorophyll content

Received for publication 4 Apr. 2011. Accepted for publication 30 May 2011.

This work was supported by grants from the National High Technology Research and Development Program of China (863 Program) (No. 2006AA10A311), the Shanghai Agricultural Achievements Transformation Program (No. 093919N0600), and the Shanghai Leading Academic Discipline Project (No. B209).

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was calculated according to Lichtenthaler (1987).

Assay of antioxidant enzymes. To extract antioxidant enzymes, 0.5 g fresh leaves were ground in liquid N₂ and homogenized with 5 mL of 0.1 M phosphate buffer (pH 7.8) containing 1 mM EDTA-Na₂ and 1% (w/v) polyvinylpyrrolidone. After centrifugation at 12,000 g_n at 4 °C for 20 min, the supernatant was used for the analysis of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD).

SOD activity was determined following the method described by Giannopolitis and Ries (1977). Reaction mixture (3 mL) was mixed with 50 mM phosphate buffer (pH 7.8), 20 μM riboflavin, 13 mM methionine, 75 μM nitroblue tetrazolium chloride (NBT), 10 μM EDTA-Na₂, and 0.1 mL enzyme extract. The reaction solution was exposed to 500 μmol·m⁻²·s⁻¹ light intensity for 20 min. One unit of SOD [unit·g⁻¹ fresh weight (FW)] activity was defined per gram of fresh leaves causing 50% inhibition of the reduction of NBT at 560 nm.

CAT activity was analyzed according to the KMnO₄ titration method (Kar and Mishra, 1976). Reaction mixture (5 mL) was mixed with 0.3 mM phosphate buffer (pH 6.8), 0.1 mM H₂O₂, and 0.1 mL enzyme extract. The reaction was stopped by adding 10 mL 2% (v/v) H₂SO₄ after 3 min. The residual H₂O₂ in the solution was titrated against 0.01 M KMnO₄. CAT activity was expressed as milligrams H₂O₂ reduced per gram of fresh leaves in 1 min (mg H₂O₂/g FW/min).

Peroxidase activity was determined using the guaiacol oxidation method (Chance and Maehly, 1955). Reaction mixture (3 mL) was mixed with 0.01 M phosphate buffer (pH 6.4), 8 mM guaiacol, 0.1 mL enzyme extract, and 2.75 mM H₂O₂. After H₂O₂ was added, the increase in absorbance was recorded at 470 nm every 10 s for the first 60 to 80 s with the spectrophotometer. One unit of POD activity was defined per gram of fresh leaves that caused a change of 0.01 in absorbance in 1 min (unit/g FW/min).

Determination of lipid peroxidation. The level of lipid peroxidation in the leaves was measured in terms of malondialdehyde (MDA) content, which was determined by the thiobarbituric acid reaction (Heath and Packer, 1968). Fresh leaves (0.5 g) were ground in liquid N₂ and homogenized with 5.0 mL of 10% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10,000 g_n at 4 °C for 10 min. The supernatant (2 mL) was mixed with 4 mL of 0.5% 2-thiobarbituric acid and heated at 95 °C for 30 min. After centrifugation at 10,000 g_n for 10 min, the absorbance of the supernatant was measured at 532 and 600 nm. The concentration of MDA was calculated using the extinction coefficient of 155 mm/cm.

Measurements of leaf photochemical efficiency and photosynthesis. Leaf photochemical efficiency of watermelon seedlings during storage was estimated by measuring chlorophyll fluorescence, Fv/Fm (ratio of variable to the maximal chlorophyll fluorescence) using

a pulse-modulated fluorometer (Model OS1-FL; Opti-Sciences, Hudson, NH) after 30 min dark adaptation.

Net photosynthetic rate and g_s of watermelon seedlings after transplanting were measured using a portable photosynthesis apparatus (Model Ciras-2; PP Systems, Hitchin, U.K.) with a quartz halogen light source at 500 μmol·m⁻²·s⁻¹ PAR. Measurements of the photochemical efficiency and photosynthesis were taken from fully expanded leaves of five randomly selected seedlings of each treatment.

Statistical analysis. The experiment was considered to be a randomized block design. Data were analyzed using analysis of variance according to the general linear model procedure by SAS software (Version 8.1; SAS Institute Inc., Cary, NC). Differences between treatments were assessed by Fisher's protected least significance difference test at the 0.05 *P* level.

Results and Discussion

Changes of starch and soluble sugar contents during storage. Because of the interruption of photosynthesis in the darkness, the carbohydrate reserves in the seedlings are important for growth during storage (Wilson et al., 1998). Nie et al. (2010) reported that grafted watermelon seedlings had better photosynthetic capacity as a result of their higher root absorption capacity. More photosynthate accumulated in the form of starch was found in the leaves of grafted watermelon seedling compared with non-grafted ones before storage (0 d) (Fig. 1A). However, there was no significant difference in soluble sugar content between grafted and non-grafted seedlings (Fig. 1B). After 2 d of storage, both starch and soluble sugar contents decreased. No significant difference in starch content between treatments was observed after 2 d, 4 d, and 6 d of storage, but the soluble sugar content of the grafted seedlings was significantly higher than that of non-grafted ones (Fig. 1). During storage, grafted seedlings degraded more starch than non-grafted seedlings not only for respiration and growth (Sato et al., 2004), but also for the maintenance of a higher level of soluble sugar. As an important osmoregulation substance in plants, soluble sugar may enhance tolerance against low temperature (Ma et al., 2009) and affect the survival ratio and photosynthetic recovery of seedlings after transplanting (Wilson et al., 1998).

Antioxidant enzyme responses and lipid peroxidation during storage. Low temperature and darkness can trigger reactive oxygen species (ROS) accumulation in plant tissues (Kanazawa et al., 2000; Liu et al., 2003). Excess ROS should be scavenged in time or it will result in lipid peroxidation and the breakdown of membrane integrity. Antioxidant enzymes participate in scavenging the excess ROS in plants. SOD catalyzes the dismutation of O₂⁻ (superoxide) into O₂ and H₂O₂ and is regarded as the first line of defense against ROS (Alscher et al., 2002).

Fig. 1. Comparisons of starch (A) and soluble sugar contents (B) of non-grafted watermelon (*Citrullus lanatus* 'Zaojia 84-24') seedlings and grafted watermelon seedlings with pumpkin (*Cucurbita moschata* 'Zhuangshi') rootstocks during 15 °C storage in darkness. Asterisk indicates significant difference between treatments on a given day (*P* = 0.05).

POD and CAT contribute to the decomposition of H₂O₂ (Toivonen and Sweeney, 1998). In this study, low-temperature storage in darkness dramatically affected the activities of antioxidant enzymes. SOD (Fig. 2A) and POD (Fig. 2B) activities of grafted seedlings were significantly higher than non-grafted ones after 4 d of storage, and a significant difference between treatments in CAT activity (Fig. 2C) was observed after 2 d of storage. During storage, grafted watermelon seedlings showed a higher antioxidant capacity, which suggests that they tolerate the storage conditions better than non-grafted ones. A recent study on cucumber (*Cucumis sativus* L.) reported grafting enhanced the gene expression of SOD and CAT under low temperature, which provided grafted cucumber seedlings better tolerance against low temperature stress (Gao et al., 2009).

As a product of membrane peroxidation, MDA content relates to the extent of damage by ROS and is a direct reflection of membrane integrity (Zhuang et al., 1995). McKay and Mason (1991) indicated that the membrane integrity of skita spruce [*Picea sitchensis* (Bong.) Carr.] and douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] seedlings was an indicator of survival after storage. MDA contents of both treatments increased during low-temperature storage in darkness (Fig. 2D). After 4 d and 6 d of storage, MDA content of grafted seedlings was significantly lower than non-grafted ones, which indicated less lipid peroxidation in grafted seedlings. The result was consistent with the activities of antioxidant enzymes (Fig. 2A–C).

Changes in chlorophyll content and chlorophyll fluorescence during storage. In the absence of light, chlorophyll in seedlings and leafy vegetables is susceptible to degradation (Toivonen and Sweeney, 1998). Thus, etiolation is often observed during storage, which affects negatively seedling quality (Ning et al., 2006a). After 6 d of storage, a significant difference in chlorophyll content was observed between treatments (Fig. 3A). ROS is regarded as an important factor to accelerate chlorophyll loss (Zhuang et al., 1995). Rapid degradation of chlorophyll of non-grafted watermelon seedlings might be the result of their weak ROS scavenging activity.

Fv/Fm is the maximum photochemical efficiency of photosystem II (PSII) and represents the function of PSII. Because PSII is sensitive to environmental stresses such as low temperature and darkness, Fv/Fm is used for evaluating the postharvest quality of leafy vegetables and the transplanting survival of tree seedlings after removal from storage (Ferrante and Maggiore, 2007; Perks et al., 2004). As shown in Figure 3B, low-temperature storage in darkness reduced the Fv/Fm of watermelon seedlings, and a significant difference in Fv/Fm between treatments was observed after 4 d of storage, which indicated the damage extent to PSII in grafted seedlings was less than that of non-grafted ones. This suggests that the grafted watermelon seedlings have a higher tolerance to low temperature and darkness.

Photosynthetic responses after transplanting. The purpose of seedling storage is to preserve seedling vigor and to ensure rapid regrowth (Kubota et al., 2002). The photosynthetic ability of a leaf determines the accumulation of carbohydrates, and so the photosynthetic rate is a good indicator of the growth potential of seedlings after transplanting (Noland et al., 1996). Low-temperature storage in darkness causes chlorophyll loss, lipid membrane peroxidation, and chloroplast ultrastructure breakdown (Ning et al., 2006b), which inevitably reduces the photosynthesis of the seedlings. Right after being removed from storage, some seedlings showed mild wilting. After 1 d of transplanting, the symptoms of wilting disappeared (not shown), and photosynthetic rate (Fig. 4A) and g_s (Fig. 4B) of grafted seedlings were significantly higher than non-grafted ones. It suggested stomatal closure was a limiting factor to photosynthesis of non-grafted seedlings. Although there was no significant difference in the g_s between treatments after 3 d of transplanting, the grafted seedlings maintained a higher photosynthetic rate. Stomatal factor was not a main element to affect photosynthesis at this stage. As mentioned, the reasons for the higher photosynthesis in grafted watermelon seedlings might be: 1) a higher carbohydrate reserve in the leaves of grafted seedlings contributed to photosynthesis; and 2) higher activities of antioxidant enzymes in the grafted seedlings scavenged the excess ROS, reduced the chlorophyll loss, and protected the photosynthetic system during storage. In addition, our previous study showed

Fig. 2. Comparisons of superoxide dismutase (SOD) activities (A), peroxidase (POD) activities (B), catalase (CAT) activities (C), and malondialdehyde (MDA) contents (D) of non-grafted watermelon (*Citrullus lanatus* 'Zaojia 84-24') seedlings and grafted watermelon seedlings with pumpkin (*Cucurbita moschata* 'Zhuangshi') rootstocks during 15 °C storage in darkness. Asterisk indicates significant difference between treatments on a given day ($P = 0.05$).

that the grafted ones had a stronger root system and quicker root regeneration after transplanting compared with the non-grafted watermelon seedlings (Ding et al., 2009), which could supply enough water to maintain a high photosynthetic rate (Mena-Petite et al., 2003).

In conclusion, grafted watermelon seedlings had better storability in the conditions of low temperature and darkness compared with non-grafted ones, which was the result of a higher carbohydrate accumulation before

Fig. 3. Comparisons of chlorophyll contents (A) and leaf photochemical efficiency (B) of non-grafted watermelon (*Citrullus lanatus* 'Zaojia 84-24') seedlings and grafted watermelon seedlings with pumpkin (*Cucurbita moschata* 'Zhuangshi') rootstocks during 15 °C storage in darkness. Asterisk indicates significant difference between treatments on a given day ($P = 0.05$).

Fig. 4. Comparisons of net photosynthetic rate (A) and stomatal conductance (g_s) (B) of non-grafted watermelon (*Citrullus lanatus* 'Zaojia 84-24') seedlings and grafted watermelon seedlings with pumpkin (*Cucurbita moschata* 'Zhuangshi') rootstocks after transplanting. Different letters above the columns indicate significant differences between treatments on a given day ($P = 0.05$).

storage, a better tolerance against low temperature and darkness during storage, and a faster recovery after transplanting.

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