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Hypoxia tolerance and adaptation of anaerobic respiration to hypoxia stress in two *Malus* species

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ABSTRACT

Seedlings of two *Malus* species (*M. hupehensis* and *M. toringoides*) were hydroponically grown in normoxic and hypoxic nutrient solutions to determine their hypoxia tolerance and adaptation of root anaerobic respiration to hypoxia stress. The growth of both species was inhibited under hypoxia stress. During hypoxia stress, the generation of superoxide anion radical ($O_2^{\bullet-}$) and content of hydrogen peroxide (H_2O_2) in roots were significantly increased in both species with similar trends, which led to an increase of malondialdehyde (MDA) content and relative membrane permeability (RMP). The degree of growth inhibition and the levels of $O_2^{\bullet-}$, H_2O_2, MDA and RMP were greater in *M. toringoides* than in *M. hupehensis*. Under hypoxia stress, pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities in roots were increased during the first 12 days of hypoxia stress and then gradually decreased in both species. The contents of acetaldehyde, alcohol and lactate in roots were also increased and showed similar trends as the activities of anaerobic respiration enzymes. The increases in PDC and ADH activities and lactate content in *M. hupehensis* under hypoxia conditions were greater than those of *M. toringoides*, but alcohol and acetaldehyde contents showed opposite trends. These data suggest that *M. hupehensis* is more tolerant of hypoxia and had less damage from oxidative stress than *M. toringoides* under hypoxia stress. The capability for anaerobic respiration is up-regulated in roots of *Malus* in response to hypoxia stress to minimize damage, and the higher hypoxia tolerance of *M. hupehensis* may be partly due to the higher enzyme activity of PDC, ADH and LDH and lower accumulation of acetaldehyde and alcohol.

1. Introduction

A well-oxygenated root-zone environment is essential for a healthy root system, but frequently they experience root-zone hypoxia mainly due to soil waterlogging, soil compaction, over-irrigation or poor drainage (Drew, 1997; Garnczarska and Bednarski, 2004; Mainiero and Kazda, 2004; Wang et al., 2009). Oxygen deprivation results in arrest of aerobic respiration, leading quickly to an energy deficit in plants (Horchani et al., 2008). Hypoxia stress is considered as one of the main environmental factors limiting plant growth and yield worldwide, especially in higher rainfall region areas. Previous studies have shown that different plants have different abilities to tolerate hypoxia, even for cultivars belonging to the same plant species but with different genotypes (Setter et al., 1994; Methode and Larry, 1999; Kato-Noguchi and Morokuma, 2007).

Most environmental stresses including hypoxia result in the production of reactive oxygen species (ROS) in plants, causing oxidative stress (Mittler et al., 2004; Garnczarska and Bednarski, 2004). ROS such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are all very reactive and cause severe damage to membranes (Bowler et al., 1992; Foyer et al., 1997). Numerous studies have shown that hypoxia stress triggers the formation of ROS and induces oxidative stress in plants (Geigenberger, 2003; Garnczarska and Bednarski, 2004; Bai et al., 2008). Under hypoxia conditions, the induction of fermentation metabolism is regarded as an adaptive phenomenon to maintain the capacity for ATP synthesis (Davies, 1980; Kennedy et al., 1992; Good and Muench, 1993). The main end product of fermentation metabolism in plants is ethanol, and the two enzymes alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC), involved in the ethanol biosynthetic pathway, are induced in such conditions (Kennedy et al., 1992; Rivoal and Hanson, 1994).

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Anoxia tolerance of plants was also associated with greater concentrations of ATP and total adenylates and with sustained levels of ADH and PDC activities (Davies, 1980; Jackson et al., 1982; Drew, 1997). In maize, rice and *Arabidopsis*, ADH and PDC loss-of-function mutants rapidly succumbed to oxygen deprivation, confirming the importance of alcohol fermentation for stress tolerance (Kennedy et al., 1992; Rahman et al., 2001). However, the anaerobic respiration response to hypoxia conditions can be different due to species and genotype diversity.

Apple (*Malus*) is one of the most economically important fruits worldwide. But apple tree often encounters root-zone hypoxia stress in production. *Malus hupehensis* and *Malus toringoides* are frequently used as rootstocks for apple cultivation in China. Previous studies showed that *M. hupehensis* from a higher rainfall region was more tolerant to hypoxia than *M. toringoides* from a low rainfall region (Bai et al., 2008). However, hypoxia tolerance and adaptation of both *Malus* species are not well known. In this study, we compared the growth, hypoxia injury and the anaerobic respiration metabolism of *M. hupehensis* and *M. toringoides* under normoxic and hypoxic conditions to investigate the difference in hypoxia tolerance and adaptation of anaerobic respiration.

2. Materials and methods

2.1. Plant material and experimental design

M. hupehensis (Chinese name is Pingyitiancha, P) and *M. toringoides* (Chinese name is Bianyehaitang, B) originated from different climate regions in China. The experiment was conducted at the Northwest A & F University, Yangling (34°20′N, 108°24′E), China. *M. hupehensis* and *M. toringoides* seeds were stratified in sand for 50 days at 0–4 °C and relative humidity of 60–70%. Three germinated seeds were planted in each plastic pot (12 cm × 12 cm) filled with sand. The plastic pots were then placed in a greenhouse under natural light and temperature conditions. From the 2nd true-leaf stage, the seedlings were watered with 1/2 Hoagland nutrient solution (pH 6.5 ± 0.1) every other day. Forty days later, seedlings with similar size (7–8 leaves, about 5 cm in height) were transferred into plastic tubs (52 cm × 37 cm × 15 cm, 50 seedlings/tub) containing 20 L 1/2 Hoagland nutrient solution. The seedlings were grown in a growth chamber, in which the temperature was controlled to 20–25 °C in the day and 15–20 °C at night and the light intensity was 140–160 μmol m^{-2} s^{-1} with a light period of 14 h. The solution was continuously aerated with an air-pump (20 min aeration per hour). Seedlings were cultured for 10 days so that these plants could adapt to ambient culture conditions.

Treatments were initiated after 10 days of solution culture in the growth chamber. A total of 400 plants were used in one-time experiment (normoxic and hypoxia treatment) for each species. (1) Control (normoxic): the nutrient solution was continuously aerated with an air-pump and maintained dissolved oxygen (DO) concentration at 8.0–8.5 mg L^{-1} by a DO controller (FC-680, Corporation of Super, Shanghai, China). (2) Hypoxia: aeration with compressed air was substituted by N_2 to maintain DO concentrations at 1.5–2.0 mg L^{-1} using the other DO controller. Each treatment was performed in 4 replicates and there are 50 seedlings used for each treatment in one replicate. At 0, 4, 8, 12, 16 and 20 days of hypoxia treatment, root samples were collected, immediately frozen in liquid nitrogen, then stored at −70 °C until use.

Each treatment was performed in 4 replicates and there were 50 seedlings used.

2.2. Growth measurements

At 20 days of treatment, plant height and root length were measured with a ruler and new leaf number was manually counted on 10 plants per treatment, and then each plant was divided into shoot and root. All the tissues were oven-dried at 70 °C for at least 72 h and then weighed.

2.3. Assay of $O_2^{\bullet-}$ generation rate

Assay of $O_2^{\bullet-}$ generation rate followed Elstner and Heupel (1976) with some modifications. Root tissue (1 g) was ground in 65 mM phosphate buffer (pH 7.8) and centrifuged at 5000 × *g* for 10 min. Supernatant of 1 mL was mixed with 65 mM phosphate buffer (pH 7.8) and 10 mM hydroxylamine hydrochloride, and placed at 25 °C for 20 min. Then 17 mM sulfanilamide and 7 mM α-anaphthylamine were added to the mixture. The absorbance of the solution at 530 nm was measured after standing for 20 min at 25 °C. A standard curve with nitrogen dioxide radical (NO_2^-) was used to calculate the $O_2^{\bullet-}$ generation rate.

2.4. Assay of H_2O_2 content

The H_2O_2 content was determined according to a method described by Patterson et al. (1984). Frozen roots were homogenized in cold acetone in the ratio of 1 g samples to 2 mL ice-cold acetone. Titanium reagent (2% $TiSO_4$) was added to a known volume of extract supernatant to give a Ti concentration of 2%. The Ti–H_2O_2 complex, together with unreacted Ti, was then precipitated by adding 0.2 mL 17 M ammonia solution for each mL of extract. The precipitate was washed five times with ice-cold acetone by resuspension, drained, and dissolved in 2 M H_2SO_4 (3 mL). The absorbance of the solution was measured at 410 nm against blanks, which had been prepared similarly but without plant tissue.

2.5. Assay of MDA content

Malondialdehyde (MDA) content was measured by the method of Heath and Packer (1968). Root tissue (0.5 g) was homogenized in 0.3% 2-thiobarbituric acid (TBA) and 10% trichloroacetic acid. After heating for 30 min at 100 °C, the mixture was centrifuged at 10,000 × *g* for 10 min. Absorbance of the supernatant was measured at 532 and 600 nm. MDA content was calculated using extinction coefficient = 155 mM^{-1} cm^{-1}.

2.6. Assay of RMP

RMP was measured following the method described by Dionisio-Sese and Tobita (1998). Roots of equal size and weight were placed in test tubes containing 10 mL distilled water. The tubes were incubated in a water bath at room temperature for 2 h and the initial electrical conductivity of the medium (L_1) was analyzed using an electrical conductivity analyzer (DDS-307, Shanghai Precision Scientific Instrument Co., Ltd., China). The samples were autoclaved at 100 °C for 20 min to release all electrolytes; cooled to 25 °C, and then the final electrical conductivity (L_2) was measured. RMP was calculated using the formula: RMP = $L_1/L_2 \times 100$.

2.7. Extraction and assay of PDC, ADH and LDH

All procedures were carried out at 0–4 °C. The extraction buffer contained 50 mM Tris–HCl (pH 6.8), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 15% (w/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol-bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA), 0.1 mM phenylmethyl sulfanyl fluoride (PMSF) (Mustroph and Albrecht, 2003). Root sample (0.5 g fresh weight) was ground in a mortar for 2 min using the extraction buffer (3 mL) and a small amount of quartz sand,