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Dissecting tissue- and species-specific responses of two *Plantago* species to waterlogging stress at physiological level

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ABSTRACT

Plantago fengdouensis is an endangered plant native to islands along the Yangtze River in the Three Gorges Reservoir of China, and *P. asiatica* is a weed mainly distributed in temperate zone of Asia. Because of contrasting distribution, tissue- and species-specific responses to waterlogging stress of these two accessions were tested in term of changes of reactive oxygen species (ROS) accumulation, membrane lipid peroxidation, free proline, and antioxidant defense systems. Our results suggested that the roots of *P. fengdouensis* had better tolerance to waterlogging stress than that of *P. asiatica*, while the leave of *P. fengdouensis* were more sensitive to waterlogging stress than that of *P. asiatica* in term of contents of hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH), and malondialdehyde (MDA). The roots of two *Plantago* species could more efficiently accumulate reduced glutathione (GSH) than leaves under waterlogging conditions. The leaves and roots of *P. fengdouensis* were more positive responses to waterlogging stress those of *P. asiatica* in term of GSH. *P. fengdouensis* were more tolerant to waterlogging stress in term of free proline levels and activities of catalase (CAT), ascorbate peroxidase (APx), and glutathione reductase (GR) than *P. asiatica*, especially in root systems. The root systems of *P. fengdouensis* had more tolerant to waterlogging stress than that of *P. asiatica*, which may be positively related to its natural inhabiting conditions. Our results may be used as indicators of reconstruction of the *ex situ* populations of the conserved *P. fengdouensis*.

1. Introduction

Plantago L. is the largest genus of Plantaginaceae, and comprises about 270 species in the world, and including 20 in China. The aerial parts and seeds of several *Plantago* species have been widely used as drugs and food due to its considerable bioactivity (Beara et al., 2012; Li et al., 2009; Janković et al., 2012; Nishibe et al., 1995). *Plantago asiatica* L. (*P. asiatica*), a weed mainly distributed in temperate zone of Asia, has a wide altitudinal range of habitats, with the highest habitation at 2600 m elevation (Murai et al., 2009). The phytochemical components in *P. asiatica* have been surveyed by several groups (Nakaoki et al., 1961; Ravn et al., 1990; Nishibe et al., 1995, 2002). In contrast, *Plantago fengdouensis* (Z. E. Zhao & Y. Wang) Y. Wang & Z. Y. Li (*P. fengdouensis*), an endangered plant native to the Three Gorge Reservoir area of China, was discovered in only two small islands of two counties along the Yangtze River in the Three Gorges Reservoir of China in 2001, and identified and named in 2004 (Wang et al., 2004a,b). Its natural habitat was seasonally flooded due to summer flooding of Yangtze River, and completely and perpetually submerged after the impoundment of the Three Gorges Reservoir (Wang et al., 2007).

Although a large number of *Plantago* species have been studied for the purpose of phytochemical analysis (Li et al., 2009; Janković et al., 2012; Nishibe et al., 1995; 2002), only few publications addressed the eco-physiological responses of *Plantago* species to abiotic stress. For example, the altitude variation had different effects on antioxidant system in leaves and in roots of *P. major* (Ren et al., 1999), and the ozone (O_3) exposure resulted in a reduction in biomass and a decrease in the number of seeds production in *P. major* (Zheng et al., 2000). Under elevated CO_2 condition, the biomass and total phenolic content of *P. maritima* increased, while protein content and the maximum carboxylation rate of Rubisco decreased (Davey et al., 2007). *P. algarbiensis* and *P. almogravensis* could efficiently accumulate aluminum from soil, especially in root tissues, and aluminum uptake was accompanied by

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substantial increases of citric, oxalic, malonic and fumaric acids contents in the plantlets of either species (Martins et al., 2013).

Waterlogging often occurs in many parts of the world every year. It often disturbs the plant growth, development, production, and many physiological functions, e.g., inhibition of photosynthesis and accumulation, increases of reactive oxygen species (ROS) production like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), and occurrence of membrane lipid peroxidation (Alhdad et al., 2013; Candan and Tarhan, 2012; Yang et al., 2011). Accordingly, in order to cope with oxidative damages and membrane lipid peroxidation, plants possess a suite of antioxidant enzymatic system and produce some non-enzymatic components like glutathione and free proline to modulate the ROS levels (Alhdad et al., 2013; Candan and Tarhan, 2012; Erkan et al., 2008; Smirnoff and Cumbes, 1989; Yang et al., 2011; Yin et al., 2009c). Perennial plants inhabiting floodplains (or river islands) cannot escape from the flooding conditions and have to endure the occurrences of waterlogging (even flooding) stress. Accordingly, these plants often develop certain phenotypic plasticity and adaptive plasticity to cope with these stresses including adaptive adjustments in biomass allocation and life cycle (Chen and Xie, 2007; Pezeshki, 2001). The present study examined the hypothesis that *P. fengdouensis* originally inhabiting islands along the Yangtze River was more tolerant to waterlogging stress than *P. asiatica* widely distributing in Asia, especially in root systems. In addition, we also examined the hypothesis that tissue-specific responses to waterlogging stress between leaves and roots of *Plantgo* species. These analyses on physiological responses of *P. fengdouensis* to waterlogging stress may be used as indicators of reconstruction of the *ex situ* populations of the *in situ* conserved *P. fengdouensis*.

2. Materials and methods

2.1. Plant materials and experimental design

The mature seeds of *P. fengdouensis* and *P. asiatica* were collected from their maternal plants grown in Wuhan Botanical Garden, Chinese Academy of Sciences in August, 2012. These seeds were used for germination in a greenhouse, which were maintained at 25–28 °C average temperature and 70–85% relative humidity. After germinating and growing for about 1 month, the seedlings were transplanted in pots (15 × 12 × 14 cm, length × width × height) filled with homogenized soil, one seedling per pot. After growth for another month in pots, the healthy and uniform seedlings were chosen from each species for waterlogging experiments. A completely randomized design with two factors (species and watering regime) was employed. The seedlings of each species were allocated randomly to two different watering regimes as follows: a well-watered treatment and a waterlogging treatment. Six replications, each with five seedlings, were used for each treatment. In the well-watered treatment, the pots were watered excessively every day. In the waterlogging treatment, all pots were flooded by standing in 108 × 54 × 25 cm (length × width × height) containers filled with tap water to 2.5 cm above the level of the soil surface. After 20 days treatment, the fresh leaves and roots were harvested and frozen in liquid N immediately for experimental analyses.

2.2. The morphological traits and biomass assay

At the end of experiments, the waterlogging and controlled plants of each species were collected randomly for pictures taking by a digital camera. In addition, six submerged and control seedlings of each species were harvested and separated into below-ground parts (roots) and above-ground parts (leaves and stems), and their individual dry weights were determined after drying at 80 °C for 48 h. The ratio of below-ground biomass to above-ground biomass was then calculated.

2.3. In situ histochemical localization of H_2O_2 and O_2^-

In situ accumulation of H_2O_2 and O_2^- was detected by histochemical staining with diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively, according to Romero-Puertas et al. (2004) and Shi et al. (2010). For O_2^- detection, the leaves and roots were immersed in NBT solution (1 mg/ml) in 10 mM phosphate buffer (pH 7.8) at room temperature for 4 h. For localization of H_2O_2, the leaves and roots were incubated in DAB solution (pH 3.8, 1 mg/ml) at room temperature for 4 h. The stained samples were then transferred to 70% (v/v) ethanol to remove chlorophyll and visualize the blue and brown spots for H_2O_2 and O_2^-, respectively. The H_2O_2 and O_2^- contents were quantified as described below to confirm the staining results.

2.4. Determination of soluble protein content, ROS level, and malondialdehyde (MDA) content

For plant protein extraction, about 1 g fresh samples were ground with liquid nitrogen and then homogenized in extraction buffer (50 mM sodium phosphate buffer, pH 7.8). After centrifuging at 12000 rpm for 30 min at 4 °C, the supernatant was quantified by Bradford method (Bradford, 1976) and used for the determination of ROS level and antioxidant enzymes activities.

The detection of O_2^- was based on the procedure of Yang et al. (2011). The O_2^- content was calculated according to a standard curve and expressed as ng/g protein. The concentration of H_2O_2 was determined as described by Yang et al. (2011). The H_2O_2 level was calculated according to a standard curve of H_2O_2. The concentration of H_2O_2 was expressed as m mol/mg protein. The OH content was assayed by the plant OH ELISA Kit based on antibody–antigen–enzyme–antibody complex following the manufacturer's instruction. After examined at the absorbance of 450 nm, the OH concentration was expressed as ng/mg protein. The MDA content was extracted using thiobarbituric acid (TBA) reagent and boiled at 100 °C for 20 min as previously described by Yang and Miao (2010). After cooling to room temperature and centrifugation at 15,000 rpm for 10 min, the MDA concentrations were determined at 450, 532 and 600 nm of absorbance with a spectrometer. The MDA concentration can be estimated through the formula $C\ (\mu mol/l) = 6.45(A_{532} - A_{600}) - 0.56A_{450}$. The MDA concentration was expressed as m mol/g FW. (FW, fresh weight)

2.5. Determination of GSH and free proline content

The concentrations of GSH were assayed using the GSH Assay Kit (A006-1, Nanjing Jiancheng, China) according to the manufacturer's instructions. After examined at the absorbance of 420 nm, The GSH concentration was expressed as mg/g protein. Proline content was estimated using described method with L-proline as standard (Bates et al., 1973; Yang et al., 2011). Briefly, the plant samples (0.5 g) were homogenized in 5 ml of 3% sulphosalicyclic acid solution. After centrifugation, 2 ml supernatant, 2 ml glacial acetic acid and 2 ml 2.5% acid ninhydrin solution were added into a test tube covered with Teflon cap. Well mixed solutions were boiled at 100 °C for 40 min. After cooling to room temperature, the proline level of samples was calculated at 520 nm absorbance by making the specification curve with known concentration of L-proline. The proline concentration was expressed as μg/g FW.