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Morphological and physiological responses of two chrysanthemum cultivars differing in their tolerance to waterlogging

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ABSTRACT

Responses to waterlogging of a tolerant chrysanthemum cultivar ('53-4') were compared with those of a susceptible one ('13-13'). Just 4 days of waterlogging were enough to induce wilting and leaf chlorosis in '13-13', but there was no visual damage to the leaves of '53-4' after 8 days of treatment. After 20 days, only a small number of adventitious roots had emerged from '13-13' stems, but many vigorous adventitious roots had formed in '53-4'. Waterlogging induced increases in the activity of alcohol dehydrogenase (EC 1.1.1.1), pyruvate decarboxylase (EC 4.1.1.1) and lactate dehydrogenase (EC 1.1.1.27) in both cultivars, but the increases in '13-13' were more pronounced than in '53-4'. On the other hand, the activity of superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11) and catalase (EC 1.11.1.6) was higher in '53-4' than in '13-13'. Leaves of '13-13' had a higher content of malondialdehyde, and the amount of this stress indicator in '53-4' was stable throughout the waterlogging period. Ethylene production was enhanced by waterlogging in both cultivars, but peak ethylene production occurred 2 days earlier in the tolerant cultivar, and was 3-fold higher than in the susceptible one.

1. Introduction

Soil waterlogging inhibits crop growth and yield, primarily through initiation of hypoxia conditions. Plants respond to this stress by generating metabolic energy from fermentative glycolysis rather than from oxidative respiration (Kumutha et al., 2008; Ismail et al., 2009; Capon et al., 2009). When the supply of oxygen is limiting, respiration shifts from the aerobic to the anaerobic mode, the latter involving glycolysis and fermentation (Kato-Noguchi and Morokuma, 2007). Pyruvate decarboxylase (PDC) catalyses the irreversible conversion of pyruvate to acetaldehyde, which represents the first step in the fermentation pathway. Alcohol dehydrogenase (ADH) then converts the acetaldehyde to ethanol, and in so doing, regenerates NAD^+, a process which is thought to be the most important function of the ethanol fermentation pathway, and one which is critical for sustaining glycolysis under hypoxia (Ismond et al., 2003; Kumutha et al., 2008). Pyruvate also represents a substrate for lactate dehydrogenase (LDH), which produces NAD^+ from its conversion to lactate. The *de novo* synthesis of anaerobic proteins (ANPs) such as PDC, LDH and ADH has been documented for a number of plant species, including rice (Kato-Noguchi and Morokuma, 2007), various salt marsh grasses (Maricle et al., 2006), maize (Vodnika et al., 2009), *Arabidopsis thaliana* (Ismond et al., 2003) and pigeon pea (Kumutha et al., 2008).

Yordanova et al. (2004) have shown that root oxygen deficiency caused photoxidative damage to barley leaves via an increased generation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$), which readily attack leaf chloroplasts and lead to leaf chlorosis and senescence (Yordanova et al., 2003). In order to cope with ROS, plants possess a suite of enzymes, primarily superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). SOD catalyses the conversion of O_2^- to H_2O_2 and gaseous oxygen. The intracellular level of H_2O_2 is regulated by a number of enzymes, of which the most important are APX and CAT (Moller et al., 2007). High levels of SOD, CAT and APX are critical for the survival of tobacco (Hurng and Kao, 1994a,b), mungbean (Ahmed et al., 2002), sweet potato (Hwang et al., 2000) and rice (Ushimaro et al., 1992) under waterlogging conditions. The molecule malondialdehyde (MDA) has been associated with lipid peroxidation via an increased generation of ROS, and thus its quantification has been suggested as a general indicator for waterlogging tolerance (Wu et al., 2003; Kumutha et al., 2009).

Under hypoxia, the ability of acetyl-coenzyme A carboxylase to oxidize its substrate 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene is compromised. ACC is synthesized in anaerobic plant roots, and transported to the stem via the xylem. There it is rapidly oxidized to ethylene by the available oxygen and can thus accumu-

Abbreviations: ADH, alcohol dehydrogenase; APX, ascorbate peroxidase; CAT, catalase; LDH, lactate dehydrogenase; MDA, malondialdehyde; PDC, pyruvate decarboxylase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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late as reported in waterlogged tomato, *Rumex* spp. and *Lepidium latifolium* (Wang and Arteca, 1992; Banga et al., 1997; Chen et al., 2002). The role of ethylene in the formation of adventitious roots under waterlogging is well documented (Shiono et al., 2008; Chen et al., 2002). Formation of adventitious roots in response to ethylene has been considered to be a major adaptive mechanism of wetland plants to root damage caused by waterlogging stress (Pezeshki, 2001). Adventitious roots emerge and grow horizontally close to the water surface, and they are connected to the stem close to the site of aerenchyma formation (Suralta and Yamauchi, 2008). Hence, adventitious roots can facilitate oxygen capture of submerged tissues, alleviating the hypoxic conditions, and contributing to the recovery and maintenance of aerobic respiration in waterlogged seedlings (Jackson and Drew, 1984; Finlayson, 2005).

Chrysanthemum (*Chrysanthemum* × *morifolium*) is native to China and Japan, and is widely cultivated for ornamental purposes (Li, 1993). The majority of cultivars are rather susceptible to waterlogging damage, and respond to sustained rainfall by first wilting, then yellowing and finally rotting; however, a small number of more tolerant cultivars, which appear able to survive episodes of waterlogging, have been identified (unpublished data). The dynamics and mechanisms of action of ANPs and antioxidant enzymes in waterlogged chrysanthemum have not been explored to date, so in this research, we set out to describe the activity of a number of ANPs, antioxidant enzymes and the response to waterlogging as measured by the content of malondialdehyde in the leaf and the release of ethylene from the submerged stem. Our purpose was to uncover some of the mechanisms involved in waterlogging tolerance in chrysanthemum, as a means of generating improved selection criteria for the development of waterlogging tolerant chrysanthemum cultivars.

2. Materials and methods

2.1. Plant materials

The chrysanthemum cultivars '53-4' (waterlogging tolerant) and '13-13' (susceptible) were obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Plants were grown in pots containing a 2:1 mixture of garden soil and vermiculite without any added fertilizer, and were maintained in a greenhouse. The greenhouse conditions were: irradiance, 160 μmol m^{-2} s^{-1} PAR, 12 h photoperiod, 25 °C average temperature, and relative humidity of ~70%. Seedlings, already grown to the 8–10 node stage were selected for uniformity. The experiment was carried out as a completely randomized split-plot with three replications. The two cultivars and the five waterlogging treatments (0, 2, 4, 6 and 8 days of waterlogging) were arranged as sub-plot and main-plot, respectively. With five plants per replication, each treatment therefore involved 15 plants. A 20 days waterlogging treatment was added to investigate the effect of a more prolonged period of stress. Within each replication, the pots were flooded by standing in a 28 cm × 14 cm × 14 cm container filled with tap water to 2.5 cm above the level of the soil surface. The tap water had a pH of 7.3 and an E.C. of 0.34 dS/m. The water temperature was held at ~25 °C. Control plants remained well-watered (60% soil moisture) throughout the experiment.

2.2. Measurement of LDH, ADH and PDC activity

Root sections (3.0–5.0 cm long, weighing ~0.5 g) were harvested at the end of each waterlogging treatment, snap frozen in liquid nitrogen, ground to a powder and stored at −70 °C until use. The material was extracted in 50 mM Tris–HCl, pH 6.8, containing 5 mM $MgCl_2$, 5 mM mercaptoethanol, 15% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA and 0.1 mM pefabloc proteinase inhibitor. LDH activity was measured by spectrophotometric monitoring of NADH oxidation during the conversion of pyruvate to lactate at 340 nm in a reaction mixture composed of 0.1 M phosphate buffer, pH 7.0, 4 μM NADH, 0.24 mM pyruvate, the reaction was initiated with addition of the extract (Bergmeyer, 1983). ADH and PDC were also measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm as described by Waters et al. (1991). The ADH assay reaction mixture was 50 mM TES, pH 7.5, 2.5 μM NADH, starting with 0.125 mM acetaldehyde, and the PDC reaction mixture was 50 mM MES, pH 6.8, 25 mM NaCl, 1 mM $MgCl_2$, 0.5 mM TPP, 2 mM DTT, 0.17 mM NADH, 50 mM sodium oxamate, 10 U ADH, starting with 10 mM pyruvate. 1 U of LDH, ADH and PDC was defined as the amount of enzyme required to decompose 1 μmol of substrate per minute per g protein.

2.3. Measurement of SOD, APX and CAT activity

A sample of ~0.5 g of leaf was harvested at the end of each waterlogging treatment, and extracted according to Polle et al. (1994). The leaf material was homogenized in 10 ml, 100 mM K_2HPO_4 (pH 7.0) containing 1 mM EDTA and 1% (w/v) polyvinyl pyrrolidone (PVP-40) at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12,000 × *g* for 20 min. SOD activity was assayed by the photochemical NBT method, in which 3 ml assay mixture contained 50 mM phosphate buffer, pH 7.8, 13 mM L-methionine, 75 μM NBT, and 2 μM riboflavin. The reduction of NBT was monitored at 560 nm and an inhibition curve constructed from various volumes of extract. 1 U of SOD was defined as sufficient to inhibit the photo-reduction of NBT by 50%. APX activity was determined in a 2 ml reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and sufficient leaf extract to induce a linear decrease in absorbance at 290 nm for 60 s (Nakano and Asada, 1981). Catalase (CAT) activity was determined by tracking the consumption of H_2O_2 at 240 nm for 3 min (Aeby, 1984), in a 3 ml assay mixture of 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 μl leaf extract. 1 U SOD, CAT and APX was defined as the amount of enzyme required to degrade 0.1 μmol of substrate per min per mg protein.

2.4. Measurement of MDA content

MDA content was determined following the method described by Zhang (1992). The level of lipid peroxidation was determined from that of 2-thiobarbituric acid (TBA) reactive metabolites. Fresh leaf tissue (~0.2 g) was homogenized, extracted in 10 ml 0.25% (w/v) TBA dissolved in 10% (w/v) trichloroacetic acid (TCA). The extract was heated to 95 °C for 30 min and then cooled quickly on ice. After centrifugation at 10,000 × *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was obtained by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as nmol per g fresh weight.

All spectrophotometric analyses were conducted on a 752 UV–vis spectrophotometer ("LENG GUANG", Shanghai, China). Protein content was determined according to the method of Bradford (1976) using BSA as a standard. Specific activities were expressed as U per g protein. All the chemicals were acquired from Sigma (Sigma).

2.5. Ethylene production

Submerged stem samples were harvested at the end of each waterlogging treatment, washed and gently blotted dry with a paper towel. The material was transferred to a gas-tight 25 ml vial and held for 6 h at 25 °C. The quantity of ethylene in the head space