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Effects of cadmium stress on seed germination and seedling growth of *Elymus dahuricus* infected with the *Neotyphodium* endophyte

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Various cadmium (Cd) concentrations (0, 50, 100, 200 and 300 μmol L^{-1}) affected *Elymus dahuricus* seed germination, seedling growth, antioxidative enzymes activities (AEA), and amounts of malondialdehyde (MDA) and proline present. These influences were determined for separate *E. dahuricus* cohorts known to be either infected (E+) or non-infected (E–) by a *Neotyphodium* endophyte. Under high Cd concentrations (100, 200 and 300 μmol L^{-1}), E+ specimens showed a significantly (P<0.05) higher germination rate and index, as well as higher values for shoot length, root length and dry biomass. However, the germination rate and index, root length and dry weight did not show a significant (P<0.05) difference under the low Cd concentrations (0 and 50 μmol L^{-1}). AEA and proline content increased, as did MDA content, in the E+ (vs. E–) specimens under high Cd concentrations. There was no significant (P>0.05) difference under low Cd concentrations. Endophyte infection was concluded to be of benefit to *E. dahuricus* exposed to high Cd concentrations.

cadmium, *Elymus dahuricus*, germination, antioxidant enzyme, endophyte

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Heavy metal contamination of the land surface and groundwater due to increased industrialization and geochemical activities is a serious environmental problem that limits crop production and bioaccumulates in the food chain, threatening human health. Cadmium (Cd) is one of the most toxic environmental pollutants for plants and can interfere with numerous biochemical and physiological processes including photosynthesis, respiration, nitrogen and protein metabolism, and nutrient uptake [1,2]. Most experiments that have been conducted on zinc stress show effects on the Calvin cycle [3] or photosystem activities [4–6]. Some studies have found that plants can tolerate Cd toxicity by inducing anti-oxidative defense systems [1,2,7].

Endophytes of *Neotyphodium* species and their sexual telemorphic genus *Epichloë* have been found in many cool-season grasses (subfamily Pooideae) [8–10] and these endophytes provide grasses with a strong competitive ability due to an increased host tolerance to biotic [11–15] and abiotic [16–18] stresses.

Elymus dahuricus is a very important grass for rangeland rehabilitation in degraded grassland zones of northern China, reducing wind erosion and land desertification [19,20]. It is a perennial caespitose grass with wide geographical distribution in most of the arid and semi-arid regions of China and Mongolia [21,22]. It also can be used to promote rapid cover and establishment mixed with saline-tolerant grasses or legumes [19–21]. *E. dahuricus* establishes a symbiotic association with systemic fungal endophytes of the genus *Neotyphodium* with an incidence that can range from 0% to 100%, based on 26 surveyed populations in China [9,22]. The fungal alkaloid peramine, which is known for its deter-

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rent effects on insects, has been detected in endophyte-infected *E. dahuricus* plants [22]. *Neotyphodium* endophyte infection can promote growth and benefit to *E. dahuricus* by stimulating anti-oxidative mechanisms, decreasing reactive oxygen species (ROS) injury under drought tolerance [23]. It was also reported that endophyte infection can increase the germination of *E. dahuricus* under various osmotic pressure potentials, with increased activities of superoxide dismutase (SOD) and peroxidase (POD) [24].

Liu *et al*. [25] reported enhanced aluminum tolerance in endophyte-infected fine fescue (*Festuca* spp.), compared with non-infected plants in terms of dry matter accumulation. Copper concentration was lower in herbage of endophyte-infected Kentucky 31 tall fescue grown in greenhouse [26] and field experiments [27]. Fabien *et al*. [6] observed that infection by an endophyte can influence higher plant values for total dry weight and tiller number, indicating a conferred tolerance of ryegrass to zinc stress. Recently, Zhang *et al*. reported that endophyte infection was of benefit to the germination [28] and anti-oxidative mechanisms of *Achnatherum inebrians* under plant exposures to high $CdCl_2$ concentrations [18].

The objective of the present study was to test a hypothesized superiority for Cd stress tolerance by infected (E+) vs. non-infected (E−) *E. dahuricus*, as reflected by the parameters of germination and growth, antioxidative enzyme activities (AEA), and the amounts of malondialdehyde (MDA) and proline present.

1 Materials and methods

1.1 Seed origin

Seeds of *E. dahuricus* were collected from E+ or E− plants grown in an experimental field (104°08′E, 35°56′N, 1514 meters above sea level) established in 2004 at the College of Pastoral Agricultural Science and Technology, Lanzhou University, Lanzhou, China. After collection, the seeds were stored at a constant 5°C temperature at the Lanzhou Official Herbage and Turfgrass Seed Testing Centre, Ministry of Agriculture, Lanzhou, China.

1.2 Germination experiments

Seeds of *E. dahuricus* were surface-sterilized with 1% (w/v) mercuric chloride followed by 75% (v/v) ethanol. Seeds were thoroughly rinsed with deionized water and allowed to imbibe for 3 h. After imbibition, the seeds were placed into Petri plates containing sterile filter sheets moistened with either 2 mL of distilled water (these controls were labeled as "CK") or $CdCl_2$ (50, 100, 200 or 300 μmol L^{-1}). Germination percentages and biochemical analyses were estimated after 14 d, by observing radicle protrusion (i.e., appearance of a radicle ⩾2 mm in length) as the criterion [28]. Each treatment was repeated five times independently using 100 seeds in each replicate. Germination index (GI)= $\sum$ (GT/DT), where GT is the germination percentage on the T day, and DT is the day of germination. The length and fresh weight of shoots and roots were recorded. Dry weight (DW) was obtained after drying at 75°C until a constant weight was recorded.

1.3 Preparations and assays of antioxidant enzymes

The extraction of antioxidant enzymes was performed as described by Zhang and Nan [23]. The SOD activity was measured spectrophotometrically as described by Beyer and Fridovich [29]. The catalase (CAT) activity was assayed as reported by Clairborne [30]. The POD activity was determined by the method of Chance and Maehly [31], using guaiacol as an electron donor. The ascorbate peroxidase (APX) activity was determined according to the method of Gupta *et al*. [32] by measuring the oxidation of ascorbate at 290 nm.

1.4 MDA content

The extent of lipid peroxidation in terms of MDA formation was measured according to the method of Esterbauer and Cheeseman [33]. A sample containing 0.5 g of plant material was mixed with 5 mL of 5% trichloroacetic acid and centrifuged at 12000×*g* for 25 min. Two milliliters of supernatant was mixed with 2 mL of a 0.67% thiobarbituric acid solution and heated for 30 min at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 450, 535 and 600 nm (A_{450}, A_{532} and A_{600}) using a blank containing all of the reagents. The MDA content of the sample was calculated using the following formula:

$$C(\mu\text{mol L}^{-1})=6.45(A_{532}-A_{600})-0.56A_{450}.$$

1.5 Proline content

Proline content was determined using a colorimetric method modified from Li [34]. A fine powder of freeze-dried leaves (0.5 g) was treated with 5 mL of 3% sulphosalicylic acid and kept at 100°C for 10 min. The supernatant (2 mL) was added to 2 mL of glacial acetic acid plus 2 mL of 2.5% (w/v) acidic ninhydrin, and then heated at 100°C for 25 min. After the liquid was cooled, it was added to 4 mL toluene. The absorbance of the extract was read at 520 nm. Contents were calculated as μg g^{-1} dry matter.

1.6 Statistical analyses

The germination data were arcsine transformed before analysis. Analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL, USA) was conducted for germination percentage, germination index, shoot/root length and fresh/dry weight. Analysis of variance was also

applied to determine the significance levels of SOD, POD, CAT, APX, MDA and Proline that resulted from various treatments of E+ vs. E− specimens.

2 Results

2.1 Percentage and index of seed germination

The germination percentage of all seeds decreased with increasing Cd concentrations, but no significant (P>0.05) difference was observed between E+ and E− seeds under low concentration (LC) stress conditions (CK and 50 μmol L^{-1}). However, there were significant (P<0.05) differences between these seed cohorts under the stress of high Cd (HC) concentrations (100, 200 and 300 μmol L^{-1}) (Figure 1A).

The germination index of seeds was also determined, and no significant (P>0.05) difference was observed between E+ and E− seeds under LC stress conditions (CK and 50 μmol L^{-1}). However, there were significant (P<0.05) differences in E+ vs. E− seeds under Cd concentrations of 100, 200 and 300 μmol L^{-1} (Figure 1B).

2.2 Shoot and root length of seedlings

Shoot lengths were determined and found to decrease with increasing Cd concentrations. These were significant (P< 0.05) differences in E+ vs. E− seedlings under the all concentrations except CK (Figure 2A).

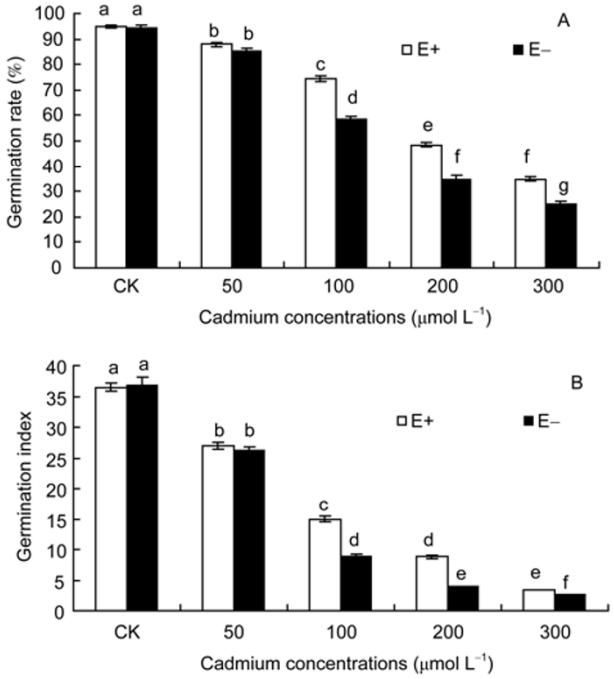

Figure 1 Seed germination of endophyte infected (E+) vs. non-infected (E−) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E− seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

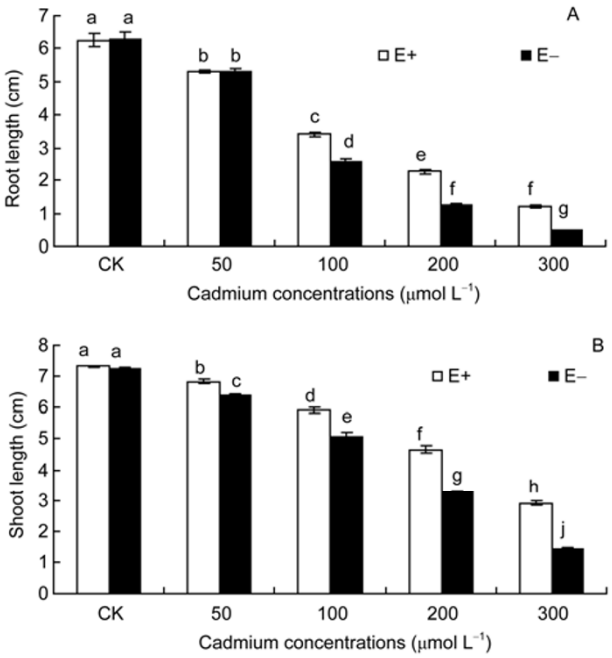

Figure 2 Seed lengths of endophyte infected (E+) vs. non-infected (E−) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E− seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

There was no significant (P>0.05) difference in root lengths between E+ and E− seeds under LC stress conditions (CK and 50 μmol L^{-1}). However, there were significant (P<0.05) differences in E+ vs. E− seedlings under Cd concentrations of 100, 200 and 300 μmol L^{-1} (Figure 2B).

2.3 Fresh and dry weights of seedlings

Fresh and dry seedling weights were found to decrease with increasing Cd concentrations. There were significant (P< 0.05) differences in fresh weights for E+ vs. E− seedlings under the all concentrations except CK. For dry weights, there was no significant (P>0.05) difference between E+ and E− seedlings under LC stress conditions (CK and 50 μmol L^{-1}). However, there were significant (P<0.05) differences in E+ vs. E− seedlings under Cd concentrations of 100, 200 and 300 μmol L^{-1} (Figure 3).

2.4 Changes in SOD activity

The activities of SOD for the plants exhibited significant (P<0.05) differences for E+ vs. E− seedlings under the all concentrations except CK (Figure 4).

2.5 Changes in POD activity

The activities of POD for the plants exhibited significant (P<0.05) differences for E+ vs. E− seedlings under the all

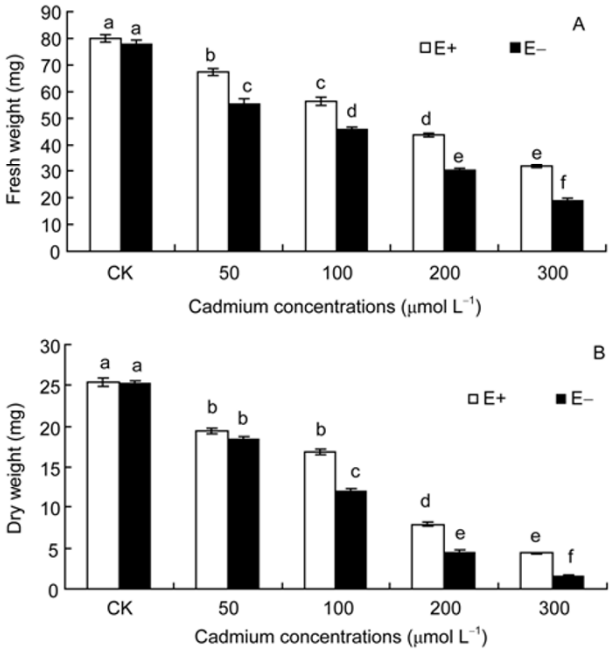

Figure 3 Seedling weights of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

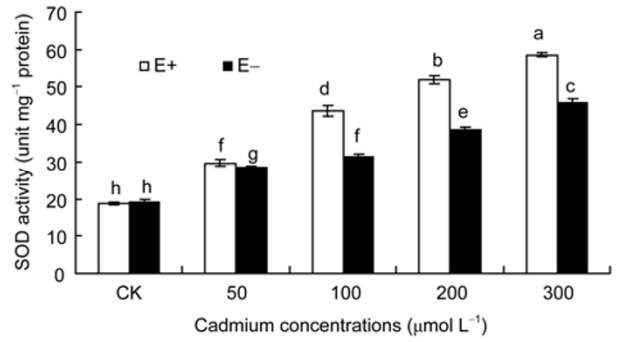

Figure 4 Variations in activity of superoxide dismutase (SOD) of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

concentrations except CK (Figure 5).

2.6 Changes in APX activity

The activities of APX for the E+ and E– plants exhibited no significant (P>0.05) difference under LC stress (CK and 50 μmol L^{-1}), but there were significant (P<0.05) increases for E+ vs. E– plants under HC stress (100, 200 and 300 μmol L^{-1}) (Figure 6).

2.7 Changes in CAT activity

The activities of CAT for the E+ and E– plants exhibited no

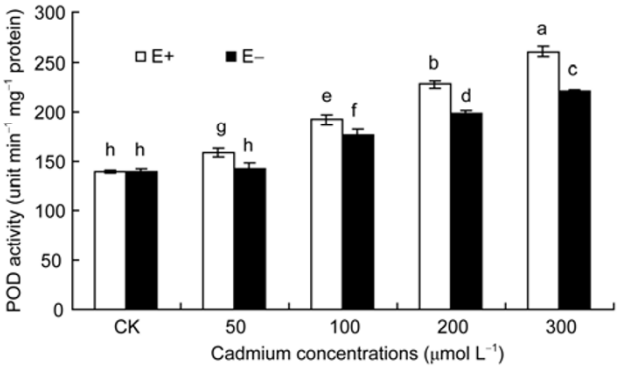

Figure 5 Variations in activity of peroxidase (POD) of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

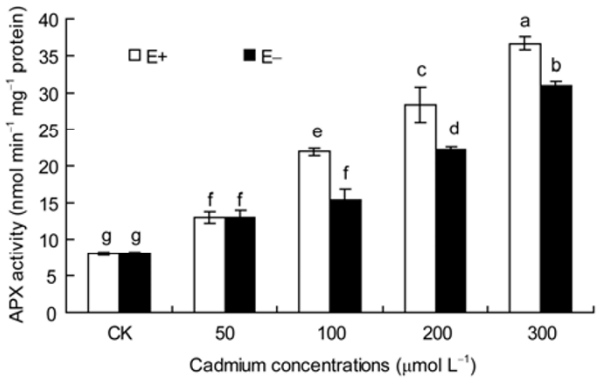

Figure 6 Variations in activity of ascorbate peroxidase (APX) of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

significant (P>0.05) differences under LC stress (CK and 50 μmol L^{-1}), but significant (P<0.05) increases were evident in E+ vs. E– plants under HC stress (100, 200 and 300 μmol L^{-1}) (Figure 7).

2.8 Changes in MDA content

The content of MDA for the E+ or E– plants exhibited no significant (P>0.05) difference under LC stress (CK and 50 μmol L^{-1}), but there were significant (P<0.05) increases in E+ vs. E– plants (Figure 8) under HC stress (100, 200 and 300 μmol L^{-1}).

2.9 Changes in proline content

Endophyte infection exhibited significant (P<0.05) effects on proline content for E+ vs. E– plants under HC stress (100, 200 and 300 μmol L^{-1}), but there was no significant

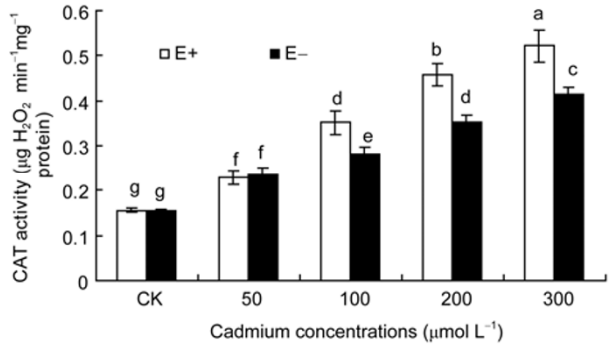

Figure 7 Variations in activity of catalase (CAT) of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

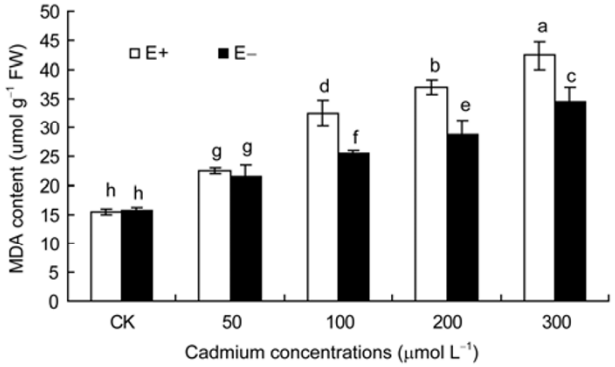

Figure 8 Variations in the content of malondialdehyde (MDA) of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

(P>0.05) difference (Figure 9) under LC stress (CK and 50 μmol L^{-1}).

3 Discussion

Seed germination is an aspect of plant fitness that may be affected by endophytes. *Neotyphodium* endophyte infection can improve plant fitness by increasing seed germination, plant length and biomass, as found in E+ vs. E– *Lolium perenne* [35], *Festuca arundinacea* [36], and *Bromus setifolius* [37], *L. multiflorum* [38], and *A. inebrians* [39]. However, research has also reported that an endophyte does not affect seed germination of *F. arizonica* [40] or *F. arundinacea* [16]. It can be argued that these inconsistencies arise from comparing experiments in which endophyte effects were confounded by the genetic origin of the host plants, and also with the conditions of seed production and after-ripening [38,39].

The present study demonstrates the effects of a *Neotyphodium* endophyte on *E. dahuricus* exposed to Cd toxicity. Exposure of E+ and E– seeds to increasing concentrations of Cd resulted in a shorter shoot length and the rapid development of leaf chlorosis, effects which have been previously reported [2,18,28,41]. Germination rates (Figure 1), shoot lengths, root lengths (Figure 2) and dry weights (Figure 3) of E+ plants were observed herein to be higher than E– plants under the Cd exposures. Zhang *et al*. [28] also reported the same results for *A. inebrians*. Some reports have shown a greater effect of Cd on root growth than shoot growth, especially at high Cd levels [18,41].

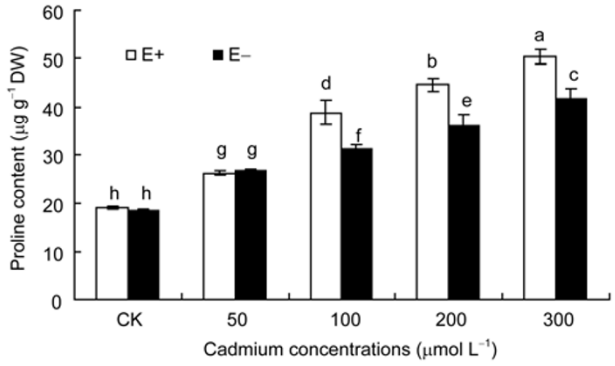

Figure 9 Variations in the content of Proline of endophyte infected (E+) vs. non-infected (E–) *Elymus dahuricus* treated with 0, 50, 100, 200 and 300 μmol L^{-1} $CdCl_2$. Tests on E+ and E– seeds (n=100) were repeated five times independently. Values are means±standard error (SE) and bars indicate SE. Differing letter pairs indicate significant difference (P<0.05).

Cd toxicity commonly represents an oxidative stress in plants by inducing formation of ROS [2,7]. Superoxide dismutase, the first enzyme in the detoxifying process of ROS, converts $O_2^{\bullet-}$ radicals to H_2O_2. The accumulation of H_2O_2 is prevented by CAT, POD and APX. Accumulation of H_2O_2 in Cd-treated plants may be the consequence of disturbing the balance between H_2O_2 production and scavenging [2,7,18,28]. The expected increase in H_2O_2, as a result of SOD inhibition, is accompanied by an increased capacity of other enzymes to decompose it. Therefore, CAT and APX play an important role in the scavenging process of H_2O_2 when coordinated with SOD activity [2].

Increased SOD, POD, CAT, and APX activities (Figures 4–7) within the leaves indicate that the *E. dahuricus* has the capacity to adapt to Cd toxicity by utilizing an antioxidant defense system, and that the endophyte enhances these enzyme activities. This increase in the activity of antioxidant enzymes (e.g., SOD, POD, CAT and APX) has also been observed after Cd exposure in other plants [2,18,28,41]. Improvement of stress tolerance is often related to an increase in activity of antioxidant enzymes [42]. One report has shown that H_2O_2 treatment can protect wheat plants from the oxidative damage of salt stress [43] and another report has also shown that alginate-derived oligosaccharides pretreatment can protect the seedling of wheat (*Triticum aestivum*) from oxidative damage by alleviating the cad-

mium toxicity [44]. Sareeta and Kavita [45] reported that increased activities of antioxidant enzymes in rice (*Oryza sativa*) were associated with the increasing Cd levels. In addition, *Vicia sativa* was shown more tolerant to Cd than *Phaseolus aureus* because of higher leaf symplastic SOD and APX activities [2]. *Rorippa globosa* leaves also had higher activity of antioxidant enzymes such as SOD and POD than that of *R. islandica* under Cd exposures [46].

Shi *et al*. [47] reported that silicon can alleviate cadmium toxicity when the Cd exposure had depressed peanut (*Arachis hypogaea*) plant growth and caused oxidative stress. Zhang *et al.* [18,28] also reported that endophyte infection was of benefit to the germination and anti-oxidative mechanisms within *A. inebrians* under plant exposures to Cd. Ren *et al.* [48] found that endophyte infection can enhance Cd accumulation in tall fescue (*L. arundinaceum*) with more tiller number and biomass, and improve Cd transport from the root to the shoot. These results suggest that endophyte-infected *E. dahuricus* confers an ecological and evolutionary advantage, as reflected by enhancement of germination and decreased ROS injury. A selection program for increased Cd tolerance in endophyte infected plants could develop more efficient strains for phytoremediation of Cd-contaminated environments [49], but the role of Cd in plant ROS production needs to be clarified by future studies.

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