Journal of Hazardous Materials 175 (2010) 703–709

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Effects of cadmium stress on growth and anti-oxidative systems in *Achnatherum inebrians* symbiotic with *Neotyphodium gansuense*

Xingxu Zhang, Chunjie Li*, Zhibiao Nan

Key Laboratory of Grassland Agro-Ecosystems, Ministry of Agriculture, College of Pastoral Agriculture Science and Technology, Lanzhou University; P.O. Box 61, Lanzhou 730000, Gansu, PR China

ARTICLE INFO

Article history:
Received 11 June 2009
Received in revised form 16 October 2009
Accepted 17 October 2009
Available online 27 October 2009

Keywords:
Cadmium
Achnatherum inebrians
Growth
Anti-oxidative enzyme
Endophyte

ABSTRACT

The effects of cadmium on biomass production and growth parameters of drunken horse grass (*Achnatherum inebrians*) over an 8-week period were determined in a controlled-environment experiment. Changes were determined for relative water content, anti-oxidative enzymes (i.e., catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX)) and for H_2O_2 content, as well as levels of proline, malondialdehyde (MDA), and chlorophylls "a" and "b" present within leaves infected with *Neotyphodium gansuense* vs. non-infected controls. Observations began 4 weeks after addition of $CdCl_2$ (0, 50, 100 and 200 μM) to the nutrient solution. Under high concentrations (100 and 200 μM) of $CdCl_2$, endophyte-infected plants produced more biomass and had higher values for plant height and tiller number compared to non-infected controls, but there was no significant difference ($P > 0.05$) under 0 and 50 μM $CdCl_2$. Anti-oxidative enzyme activities, H_2O_2 concentration, and chlorophylls "a" and "b" levels increased, but proline and malondialdehyde content declined in the infected plants vs. non-infected plants under high (100 and 200 μM) concentrations of $CdCl_2$. There was no significant difference ($P > 0.05$) under 0 and 50 μM $CdCl_2$. Endophyte infection was concluded to be of benefit to the growth and anti-oxidative mechanisms within *A. inebrians* under high concentrations exposures to $CdCl_2$.

1. Introduction

Heavy metal contamination is a serious environmental problem. Cadmium is one of the most toxic of environmental pollutants for plants and can interfere with numerous biochemical and physiological processes, including photosynthesis, respiration, nitrogen and protein metabolism, and nutrient uptake [1]. Plants can tolerate cadmium toxicity by inducing anti-oxidative defense systems [2,3].

Endophytes of *Neotyphodium* species and their sexual telemorphic genus *Epichloë* have been found in many cool-season grasses [4,5] and are associated with increased host resistance to abiotic and biotic stresses [6–8], nutrient acquisition [9] and competitive ability [10,11].

Drunken horse grass (*Achnatherum inebrians*) is an important perennial Chinese bunchgrass which usually grows on roadsides, in gullies, on shady slopes, and in the harsh conditions of the alpine or subalpine grasslands of the Qinghai-Tibetan, Tianshan and Qilian mountains in Gansu, Xinjing, Qinghai, Tibet and Inner Mongolia [12]. It could, therefore, have potential use as a sustainable soil–water conservation plant. *Neotyphodium gansuense* is a fungal endophyte symbiotic with the *A. inebrians* that is native to Gansu, China. It was described by Li et al. [12] and subsequently characterised [13]. *Neotyphodium* endophytes provide *A. inebrians* with a strong competitive ability due to an increased host tolerance to drought [14], salt [15] cold [16], pathogenic fungi [17], and pests [18,19].

Zaurov et al. [20] reported that endophyte infection can enhance the aluminum tolerance of fine fescues (*Festuca* spp.). Fabien et al. [21] demonstrated that such an infection can improve ryegrass (*Lolium perenne*) values of total dry weight and tiller number, indicating a tolerance to zinc stress. Gou [15] reported that *N. gansuense* can improve the sodium tolerance of infected (E+) *A. inebrians* compared to non-infected (E−) specimens. Wang et al. [22] also reported that an endophyte infection can improve the sodium tolerance of *Hordeum brevisubulatum*. However, no reports currently exist on interactions between cadmium, endophytes and grasses.

Thus, the present study examines the effects of *N. gansuense* on *A. inebrians* grown under cadmium-stressed or normal conditions, including effects on cadmium-influenced host growth parameters, as determined by measuring a series of physiological and biochemical indicators. The goal of this work was to better understand the physiology and possible mitigation of cadmium toxicity within *A. inebrians* due to endophyte infection.

* Corresponding author. Tel.: +86 931 8914233; fax: +86 931 8910979.
E-mail address: chunjie@lzu.edu.cn (C.J. Li).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2009.10.066

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *A. inebrians* were collected from E+ plants grown in an experimental trial plot within Xiahe county (E: 102°31′, N: 35°12′, elevation: 2931 m) in Gansu, China. These seeds had previously been stored at a constant 5 °C temperature at the Lanzhou Official Herbage and Turfgrass Seed Testing Centre, Ministry of Agriculture, Lanzhou, China.

To reduce genotypic variation between E+ and E− lines, a subset of E+ seeds was freed of the endophyte using high temperature and high humidity to yield E− seeds from the same maternal line as used by Li [23]. These E+ or E− seeds were planted into segregated pots (30 cm × 25 cm × 8 cm) filled with silt loam soil that had been sterilized in an oven at 160 °C for 6 h. E+ and E− seedlings were subsequently transplanted individually into plastic pots, placed within a constant temperature greenhouse (22 °C) with a day/night cycle of 16 h light: 8 h dark (L16:D8), and were watered, as needed.

2.2. Treatments

Pots were maintained in the greenhouse for 4 weeks, and the plants were trimmed weekly to 10 cm above the soil surface, before the $CdCl_2$ stress was imposed. At approximately 6 weeks post-sowing, 20 pots (5 replicates × 4 treatments) were randomly assigned to four $CdCl_2$ treatments (i.e., concentrations of 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution) for each of the tests.

Mature leaves were harvested 1-month later. Parts of the fresh leaves were used to extract and assay enzymes immediately. Other leaf parts were plunged into liquid nitrogen for 2 min and freeze-dried at −60 °C, then ground to a fine powder with a mortar and pestle and stored at −80 °C for later proline, malondialdehyde (MDA) and chlorophyll determinations.

2.3. Biomass production and growth

The initial heights of three labeled plants within each pot, and their respective numbers of tillers, were measured. The effects of endophyte infection and $CdCl_2$ stress on biomass production and growth parameters were then determined over an 8-week period. At the end of the trial, each harvested plant was divided into roots and above-ground parts, soil was washed from the roots with tap water, and root diameters were determined with a Vernier caliper (Mututoyo, Japan).

2.4. Relative water content

Pre-dawn relative water content (RWC) was determined on six leaves, 1-month later. Fresh weight (FW) was determined after excision, and the leaves were rehydrated in the dark for at least 18 h to determine leaf turgid-weight (TW) [24]. Dry weight (DW) was determined after drying the leaves to a constant weight in an oven at 105 °C. The RWC was calculated from the equation, RWC = 100[(FW − DW)/(TW − DW)].

2.5. Extraction and assay of enzymes

The extraction of anti-oxidant enzymes was carried out on leaf samples using methods described by Zhang and Nan [25] with minor modifications. Leaf tissues (0.5 g) were homogenized in 5 ml of 50 mM sodium phosphate buffer (pH 7 for catalase (CAT), and pH 7.8 for superoxide dismutase (SOD), peroxidase (POD) and ascorbate peroxidase (APX)) containing 1% (w/v) polyvinylpyrrolidone and 0.1 mM Na_2EDTA. The homogenate was then filtered through four layers of cheesecloth and centrifuged at 15,000 × *g* for 20 min. After centrifugation, aliquots of the supernatant were used to determine the enzyme activities and protein concentration. Extracts were prepared at 4 °C, and enzyme assays were performed at 25 °C. Catalase activity was assayed as reported by Clairborne [26]. Superoxide dismutase activity was measured spectrophotometrically as described by Beyer and Fridovich [27]. Peroxidase activity was determined by the method of Chance and Maehly [28] using guaiacol as an electron donor. Ascorbate peroxidase activity was determined according to the method of Gupta et al. [29] by measuring the oxidation of ascorbate at 290 nm. Protein concentration in each enzyme extract was measured by the method of Bradford [30]. Hydrogen peroxide was determined according to a method modified from Velikova et al. [31] with minor modifications. Leaf tissues (1.0 g) were homogenized in an ice bath with 10 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 20 min. One milliliter of supernatant was added to a solution of 1 ml of 10 mM sodium phosphate buffer (pH 7.0) plus 2 ml of 1 M KI. The photometric absorption of the reaction solution supernatant was measured at 390 nm.

2.6. Proline content

Proline content was determined using a colorimetric method modified from Li [32]. The fine powder of freeze-dried leaves (0.5 g) were treated with 5 ml of 3% sulphosalicylic acid and maintained at 100 °C for 10 min. The supernatant (2 ml) was added to a solution of 2 ml of glacial acetic plus 2 ml of 2.5% (w/v) acidic ninhydrin, and kept at 100 °C for 25 min. After the liquid was cooled, it was added to 4 ml of toluene. The photometric absorbance of the toluene extract was read at 520 nm. Contents were calculated to $\mu g/g^{-1}$ dry matter.

2.7. Chlorophyll content

Chlorophylls "a" and "b" contents was determined using a colorimetric method modified from Li [32]. A sample containing 0.2 g frozen and ground plant tissue was extracted in 80% acetone mixed with little calcium carbonate powder and centrifuged at 12,000 × *g* for 25 min. The absorbance of the sample was measured at 645 and 663 nm against a blank containing all the reagents, but without the sample. The chlorophyll content of the sample was calculated using the formula:

$$C\text{“a”}(\mu mol/l) = 12.72A_{663} - 2.59A_{645};$$

$$C\text{“b”}(\mu mol/l) = 22.88A_{645} - 4.67A_{663}$$

2.8. MDA content

The extent of lipid peroxidation in terms of malondialdehyde formation was measured according to a method modified from Li [32]. A sample containing 0.5 g of plant materials was mixed with 5 ml TCA (5%) and centrifuged at 12,000 × *g* for 25 min. Two milliliter of supernatant was mixed with 2 ml of a 0.67% thiobarbituric acid (TBA) solution and incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an the ice bath. Then the samples were centrifuged at 12,000 × *g* for 5 min and the absorbancy of supernatant was used for the determination of the MDA content. The value for the non-specific absorption at A_{600} was subtracted from the A_{532} reading. The amount of MDA–TBA complex (red pigment) was calculated by using the extinction coefficient for MDA of 155 mM^{-1} cm^{-1}.

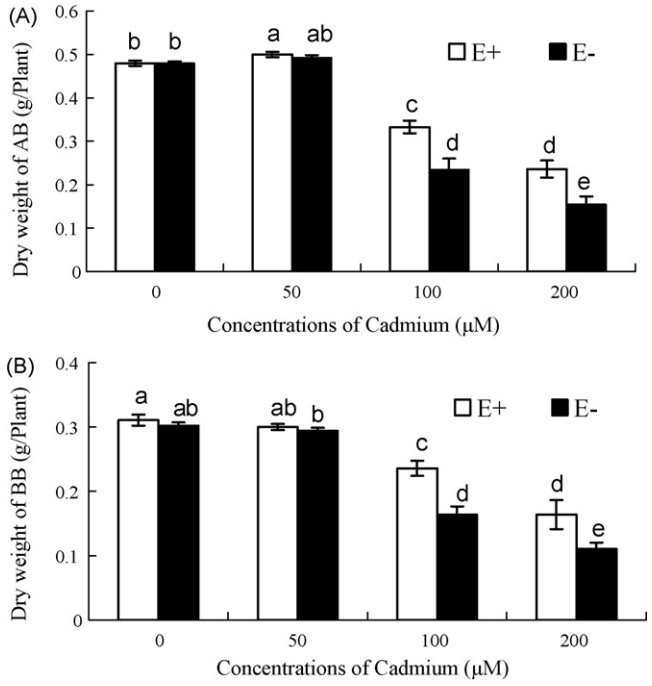

Fig. 1. Dry weight of above-ground (AB) biomass (A) and below-ground (BB) biomass (B) of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 8 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution. Endophyte-infected (E+) and endophyte free (E−) plants in each replicate were all three plants ($n = 5$). Values are means ± standard error (SE) and bars indicate SE. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

2.9. Statistical analyses

Results of the biomass and root parameters, the activities of CAT, SOD, POD and APX, and the contents of proline, MDA, and chlorophylls "a" and "b", that resulted from E+ and E− plant specimens that did undergo the various cadmium treatments, were presented as means with standard error. Statistical analyses, one-way analysis of variance (ANOVA) and Duncan's multiple range tests were performed by using the SPSS statistical software package (Ver.13.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Plant growth parameters and biomass production

Plant heights and tiller numbers were observed (Fig. 1) to be significantly higher ($P < 0.05$) for E+ specimens (relative to E− plants) growing under the high (100 and 200 μM) $CdCl_2$ conditions. However, decreased plant heights and tiller numbers were exhibited in both cases, relative to growth using 0 and 50 μM $CdCl_2$ concentrations. No differences were observed between E+ and E− plants growing under the 0 and 50 μM $CdCl_2$ conditions.

Diameter and number of E+ roots were both significantly higher ($P < 0.05$) under 100 and 200 μM $CdCl_2$ conditions vs. those of E− plants. E+ values were higher for all treatments, but endophyte infection did not have a significant effect on these two parameters under the 50 μM $CdCl_2$ condition. Significantly longer root lengths ($P < 0.05$) occurred in E+ specimens vs. E− plants under all stress concentrations (Fig. 2). However, growth was generally suppressed for all plants under the 100 and 200 μM $CdCl_2$ conditions.

No significant ($P > 0.05$) difference was observed between E+ and E− plants under 0 and 50 μM $CdCl_2$ for above-ground and below-ground biomass production (Fig. 3A). However, there was

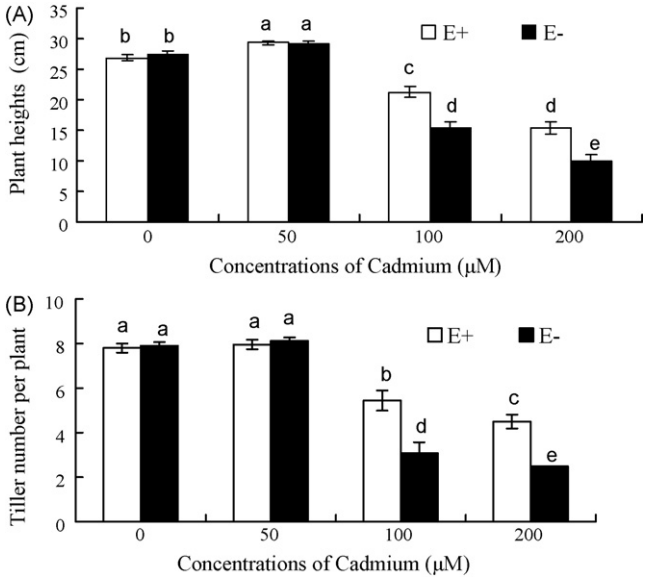

Fig. 2. Plant height and tiller number of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 8 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution. Endophyte-infected (E+) and endophyte free (E−) plants in each replicate were all three plants ($n = 5$). Values are means ± standard error (SE) and bars indicate SE. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

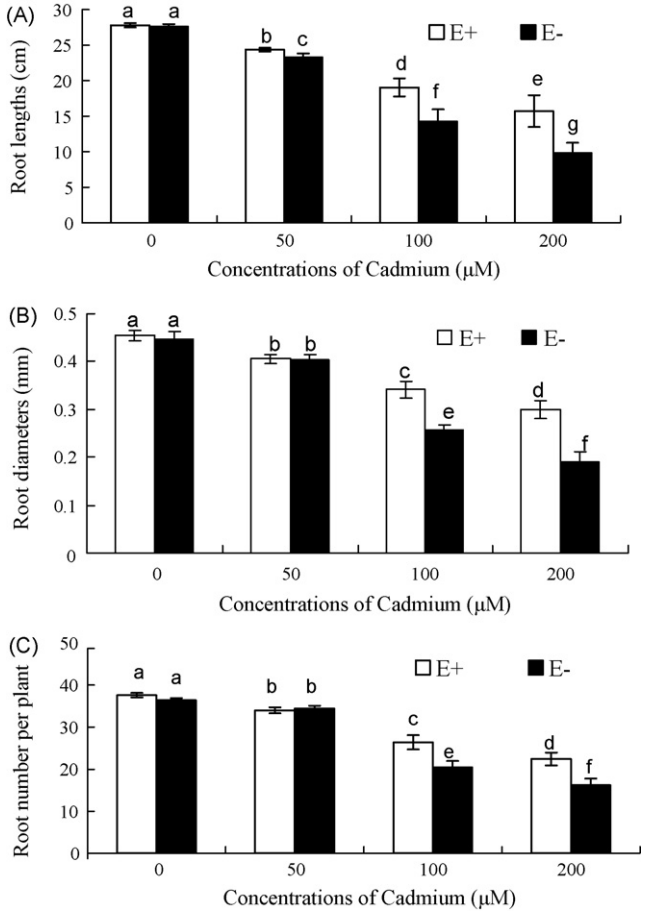

Fig. 3. Root length, root diameter and numbers of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 8 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution. Endophyte-infected (E+) and endophyte free (E−) plants in each replicate were all three plants ($n = 5$). Values are means ± standard error (SE) and bars indicate SE. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

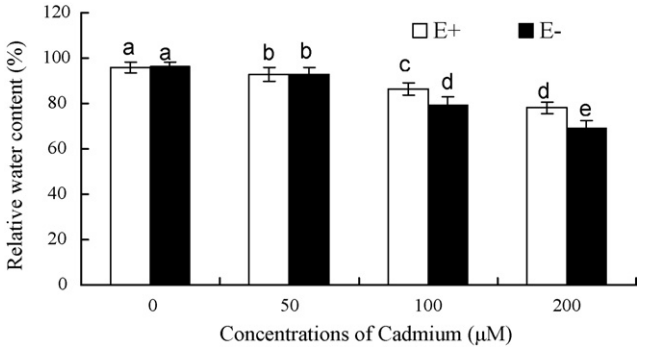

Fig. 4. Relative water content of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 4 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution. Endophyte-infected (E+) and endophyte free (E−) plants in each replicate were all three plants ($n = 5$). Values are means ± standard error (SE) and bars indicate SE. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

significantly ($P < 0.05$) more above-ground (Fig. 3A) biomass production for E+ vs. E− plants under the 100 and 200 μM $CdCl_2$ conditions, with a relative increase of 29.7% and 53.4%, respectively. There was also significantly ($P < 0.05$) greater below-ground (Fig. 3B) biomass production in E+ compared with E− plants under 100 μM and 200 μM $CdCl_2$ conditions, an increase of 30.5% and 32.5%, respectively. However, in all cases, plants grown under the 100 μM and 200 μM $CdCl_2$ conditions had overall lower biomass production than those grown under the 0 and 50 μM $CdCl_2$ conditions.

3.2. Changes in relative water content and chlorophylls of leaves

Relative water contents of leaves were also determined, and these values were observed to decrease with increasing cadmium concentrations. No significant ($P > 0.05$) difference was observed between E+ and E− plants under 50 μM stress conditions. However, there was a significant ($P < 0.05$) water increase in E+ compared with E− plants under the 100 and 200 μM $CdCl_2$ conditions (Fig. 4), although overall, relative water content was observed to decrease among all specimens with increasing concentrations of $CdCl_2$.

There were no significant effects of endophyte status on leaf contents of chlorophyll "a" under 50 μM and 100 μM $CdCl_2$ ($P > 0.05$), whereas effects of endophyte on contents of chlorophyll "a" were significant ($P < 0.05$) under 0 and 200 μM $CdCl_2$ (Fig. 5A). The concentrations of chlorophyll "b" were also determined, with E+ values being higher within all treatments. No significant ($P > 0.05$) difference was observed between E+ and E− plants under the 50 μM $CdCl_2$ condition, although there was a significant difference ($P < 0.05$) under the 100 and 200 μM $CdCl_2$ conditions, with increases of 31.9% and 31.5%, respectively (Fig. 5B). However, the presence of both chlorophylls was observed to diminish among all specimens with increasing concentrations of $CdCl_2$.

3.3. Changes in contents of proline and MDA

The proline content of E− plants was significantly ($P < 0.05$) higher than in E+ plants under all $CdCl_2$ concentrations, except the 200 μM condition (Fig. 6A). In that instance, proline content declined in E− plants, although the rising proline content trend for E+ specimens continued, this value being significantly ($P < 0.05$) higher than that of the E− plants.

A similar positive correlation between $CdCl_2$ concentrations and MDA levels was observed (Fig. 6B), although there was no crossover at 200 μM $CdCl_2$. There was significantly ($P < 0.05$) more MDA content in E+ vs. E− plants within the 50, 100 and

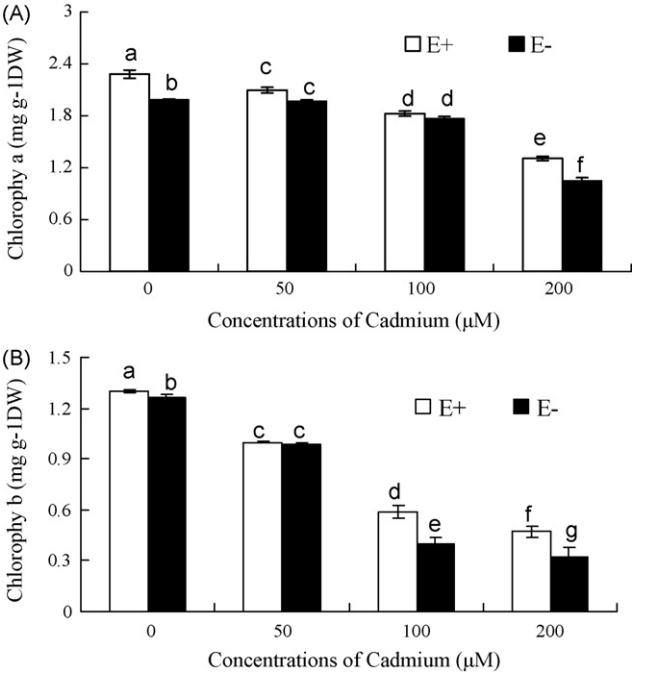

Fig. 5. Variations in content of chlorophyll a, b of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 4 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution. Endophyte-infected (E+) and endophyte free (E−) plants in each replicate were all three plants ($n = 5$). Values are means ± standard error (SE) and bars indicate SE. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

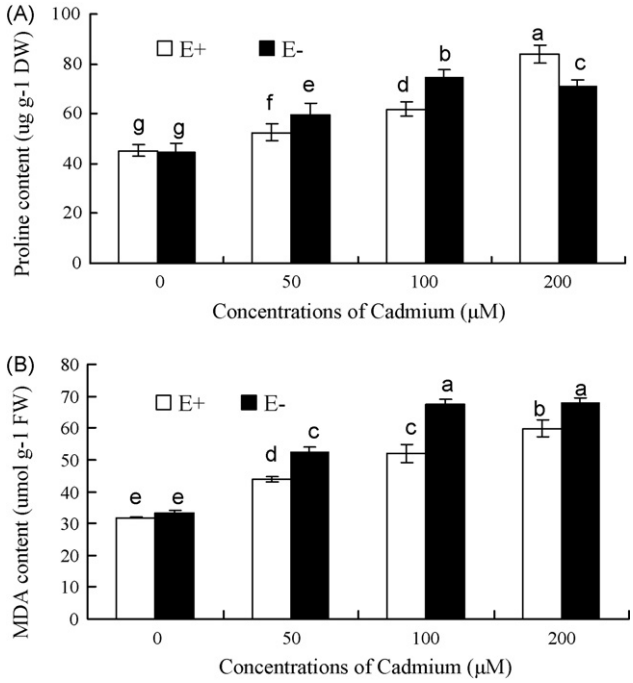

Fig. 6. Variations in content of proline and MDA of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 4 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution. Endophyte-infected (E+) and endophyte free (E−) plants in each replicate were all three plants ($n = 5$). Values are means ± standard error (SE) and bars indicate SE. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

Table 1
Variations in activities of SOD, POD, CAT, APX and content of H_2O_2 of endophyte-infected (E+) vs. uninfected (E−) *Achnatherum inebrians* after a 4 weeks treatment under 0, 50, 100 and 200 μM $CdCl_2$ in half-strength Hoagland's solution.

Concentrations (μM)	Plant	Index				
		SOD	POD	CAT	APX	H_2O_2
0	E+	29.64 ± 2.28f	381.4 ± 12.2g	0.420 ± 0.017e	25.4 ± 0.51g	35.4 ± 1.41f
	E−	28.68 ± 2.16f	380.8 ± 12.43g	0.406 ± 0.012e	24.4 ± 0.40g	35.2 ± 0.49f
50	E+	37.26 ± 2.98e	491.2 ± 12.81e	0.586 ± 0.021c	39.4 ± 2.93e	45.0 ± 1.71d
	E−	37.44 ± 0.82e	463.2 ± 16.88f	0.530 ± 0.007d	33.8 ± 1.37f	44.4 ± 0.68de
100	E+	53.54 ± 0.99c	600.8 ± 14.14c	0.826 ± 0.019a	53.6 ± 1.51c	62.0 ± 2.71b
	E−	45.42 ± 1.57d	527.4 ± 13.44d	0.618 ± 0.015c	45.2 ± 2.37d	42.8 ± 1.86e
200	E+	66.70 ± 2.62a	735.8 ± 16.28a	0.760 ± 0.012b	66.2 ± 2.58a	75.8 ± 2.80a
	E−	57.08 ± 4.58b	625.8 ± 13.59b	0.594 ± 0.012c	57.4 ± 2.51b	56.4 ± 2.68c

Values are means ± standard error (SE) and the different letters in the same row indicated significant difference at $P < 0.05$ (Duncan test).

200 μM $CdCl_2$ treatments, with increases of 16.4%, 29.5% and 13.3%, respectively.

3.4. Changes in CAT, SOD, POD, APX, activities and H_2O_2 concentration

E+ values increased for CAT activity with increasing $CdCl_2$ concentrations and were significantly ($P < 0.05$) increased compared to E− plants (Table 1).

The activities of SOD were not significantly ($P < 0.05$) different within the E+ and E− plants for 50 μM $CdCl_2$, but were significantly ($P < 0.05$) increased under 100 and 200 μM $CdCl_2$ concentrations, more so in E+ than in E− plants (Table 1).

Both POD and APX activities increased significantly ($P < 0.05$) in response to increased stress conditions (Table 1), more so in E+ than in E− plants.

The levels of H_2O_2 in the E+ and E− plants exhibited no significant difference under the 50 μM $CdCl_2$ condition, but were significantly ($P < 0.05$) increased under 100 μM and 200 μM $CdCl_2$ concentrations (Table 1), more so in E+ than in E− plants.

4. Discussion

To the best of our knowledge, the present work represents a first study on the interaction between a *Neotyphodium* endophyte, cadmium toxicity and *A. inebrians*, and demonstrates that the presence of the endophyte can apparently ameliorate the effects of cadmium toxicity.

Exposure of the experimental plants to increasing concentrations of cadmium resulted in reduction in tiller number, shoot length, and root number, length, and diameter. Occurrence of these phenomena was associated with reductions in dry matter production. The results indicate that endophyte infection is beneficial under conditions of cadmium stress to improve above-ground and below-ground biomass (Fig. 1), tiller number (Fig. 2) and root parameters (Fig. 3). The effects of this endophyte on plant growth and physiology for *A. inebrians* under NaCl stress were also reported by Gou [15] and Li et al. [33]. Zaurov et al. [20] reported that endophyte infection can enhance the aluminum tolerance, such as more biomass, of fine fescues. Fabien et al. [21] showed that an endophyte can improve higher values of total dry weight and tiller number, increasing zinc stress tolerance for ryegrass.

Melis et al. [34] indicated that cadmium toxicity causes development of reddish-brown necrotic spots on older leaves of barley (*Hordeum vulgare*) cultivars (cvs. Tokak and Hamidiye). A similar symptom was also found in shoots of *A. inebrians* within this study. In addition, RWC (Fig. 4) and chlorophylls "a" and "b" contents (Fig. 5) were reduced with increased concentrations of cadmium. Under strong drought stress, Li found greater RWC and accumulation of chlorophylls in E+ *A. inebrians* [14], with similar results being found by Gou [15]. Zhang and Nan [25] found a greater RWC and accumulation of chlorophyll in E+ vs. E− *Elymus dahuricus*. Hunt et al. [35] also found greater concentrations of chlorophyll in E+ perennial ryegrass. As chlorophyll is the basis for photosynthesis, any chlorophyll reduction in E− plants would affect their photosynthetic capacity, and indeed, stronger net photosynthesis in E+ vs. E− tall fescue has been found [35,36].

It is well known that the presence of proline may play a role in plant protection from desiccation and from the harmful effects derived from solute accumulations. In the present study, high cadmium stress conditions induced proline accumulations approximately comparable between E+ and E− plants (Fig. 6), a result which may indicate that both adapt to cadmium stress similarly. Gou [15] reported that salt stress induced more proline accumulation in E+ than in E− *A. inebrians*, and a similar result was also reported by Li [14]. However, Zhang and Nan [25] reported that water stress conditions induced proline accumulation in E+ plants comparable with E− plants. Elbersen and West [37] found lower proline concentrations in E+ tall fescue plants under field drought conditions, with proline content being higher in E+ (vs. E−) plants under the stress of higher concentrations of 200 μM $CdCl_2$. This could be interpreted as due to the more severe cadmium damage in E− plants, which could not protect themselves as effectively. However, these differing results may be attributable to the effects of various stresses on different species under dissimilar conditions.

Plants have evolved several methods to prevent damage by reactive oxygen species (ROS). One of these mechanisms is the employment of an enzymatic anti-oxidant system that includes catalase, superoxide dismutase, peroxidase and ascorbate peroxidase [38,39]. Bonnet et al. [40] reported that decreased APX activity was found in E+ ryegrass under mild, but not severe, zinc stress. Concentration of H_2O_2 and O^{2-} has been observed in some cadmium-exposed plant species [41,42]. In the present study, cadmium-induced production of H_2O_2 and O^{2-} in the leaves of *A. inebrians* was demonstrated using both histochemical and cytochemical methods. Alternatively, cadmium may also activate the O^{2-} generating membrane-bound enzyme NADPH-oxidase [43,44]. By interacting with membrane lipids and proteins, cadmium can result in structural impairments of cell membranes, with a concomitant activation of membrane-bound O^{2-} generating NADPH-oxidase [45,46]. In the roots [47] and leaves [48] of rice, O^{2-} production was increased in response to cadmium treatment, and enhanced activity of NADPH-oxidase by cadmium was suggested as a possible reason for cadmium-induced O^{2-} formation. Buckhout et al. [49] demonstrated enhanced levels of H_2O_2 production by cadmium supply to tobacco cells, and suggested that a NADPH-oxidase-like enzyme is induced by cadmium toxicity.

Phaseolus aureus has been shown to produce more H_2O_2 and O^{2-} than *Vicia sativa* exposed to cadmium [1].

In the present study, contents of H_2O_2 had higher values under cadmium stress (Table 1). These results show that cadmium stress induces intracellular oxidizing conditions leading to the production of ROS. Although possible interactions between an endophyte and elements of plant defense mechanisms are not yet well understood, it was found that activities of CAT, SOD, POD and APX were significantly increased in the E+ (vs. E−) plants under long-term cadmium stress (Table 1).

These results suggest that endophyte-infected *A. inebrians* may have an ecological and evolutionary advantage over uninfected specimens, as reflected by morphological parameters and reduced ROS injury, but the role of cadmium in plant ROS production needs to be clarified by further studies.

Acknowledgments

We wish to thank Dr. Peter Long for polishing the English and Dr. Fenqin Zhang for providing beneficial discussion of the manuscript. This research was financially supported by the National Basic Research Program of China (2007CB108902), National Key Technology R & D Program of China (2008BADB3B05) and the National Nature Science Foundation of China (30771531).

References

[1] F.Q. Zhang, H.X. Zhang, G.P. Wang, L.L. Xu, Z.G. Shen, Cadmium-induced accumulation of hydrogen peroxide in the leaf apoplast of *Phaseolus aureus* and *Vicia sativa* and the roles of different antioxidant enzymes, J. Hazard. Mater. 168 (2009) 76–84.
[2] S.D.L. Toppi, R. Gabbrielli, Response to cadmium in higher plants, J. Exp. Bot. 41 (1999) 105–130.
[3] A. Hegedus, S. Erdei, G. Horvath, Comparative studies of H_2O_2 detoxifying enzymes in green and greening barley seedlings under cadmium stress, Plant Sci. 160 (2001) 1085–1093.
[4] Z.B. Nan, C.J. Li, *Neotyphodium* in native grasses in China and observations on endophyte/host interactions, in: V.H. Paul, P.D. Dapprich (Eds.), Proceedings of Fourth International *Neotyphodium*/Grass Interactions Symposium, Soest, Germany, 2000, pp. 41–50.
[5] C.L. Schardl, A. Leuchtmann, The Epichloë endophytes of grasses and the symbiotic continuum, in: J. Dighton, J.F. White, P. Oudemans (Eds.), The Fungal Community, third ed., CRC Press, Boca Raton, 2004, pp. 475–503.
[6] B. Meister, J. Krauss, S.A. Härri, M.V. Schneider, C.B. Müller, Fungal endophyte symbionts affect aphid population size by reduction of adult lifespan and fecundity, Basic Appl. Ecol. 7 (2006) 244–252.
[7] J.P.J. Eerens, M.H.P.W. Visker, R.J. Lucas, Influence of the ryegrass endophyte (*Neotyhodium lolii*) in a cool moist environment IV. Plant parasitic nematodes, N Z J. Agric. Res. 41 (1998) 209–217.
[8] C.W. Bacon, Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue, Agric. Ecosyst. Environ. 44 (1993) 123–142.
[9] D.P. Malinowski, D.P. Belesky, G.C. Lewis, Abiotic stresses in endophyte grasses, in: A.R. Craig, P.W. Charles, E.S. Donald (Eds.), *Neotyphodium* in Cool-season Grasses, 2005, pp. 187–199.
[10] D.P. Malinowski, A. Leuchtmann, D. Schmidt, J. Nosberger, Symbiosis with *Neotyphodium uncinatum* endophyte may increase the competitive ability of meadow fescue, Agron. J. 89 (1997) 833–839.
[11] C.B. Müller, J. Krauss, Symbiosis between grasses and asexual fungal endophytes, Curr. Opin. Plant Biol. 84 (2005) 50–456.
[12] C.J. Li, Z.B. Nan, V.H. Paul, P. Dapprich, Y. Liu, A new *Neotyphodium* species symbiotic with drunken horse grass (*Achnatherum inebrians*) in China, Mycotaxon 90 (2004) 141–147.
[13] C.J. Li, Z.B. Nan, F. Li, Biological and physiological characteristics of *Neotyphodium gansuense* symbiotic with *Achnatherum inebrians*, Microbiol. Res. 163 (2008) 431–440.
[14] F. Li, Effects of endophyte infection on drought resistance to drunken horse grass (*Achnatherum inebrians*), Master thesis, Lanzhou University, China, 2007.
[15] X.Y. Gou, Effect of *Neotyphodium* endophyte on salt tolerance to drunken horse grass (*Achnatherum inebrians*), Master thesis, Lanzhou University, China, 2007.
[16] N. Chen, Genetic diversity of drunken horse grass (*Achnatherum inebrians*) and effects of its endophyte infection on cold tolerance, Master thesis, Lanzhou University, China, 2008.
[17] C.J. Li, Z.B. Nan, F. Li, Biological and physiological characteristics of *Neotyphodium gansuense* symbiotic with *Achnatherum inebrians*, Microbiol. Res. 163 (2007) 431–440.
[18] X.X. Zhang, C.J. Li, Effects on bird cherry-oat aphid resistance to drunken horse grass by *Neotyphodium* endophyte infection, in: Organizing Committee of 2008 IGC/IRC Conference (Ed.), Multifunctional Grasslands in a Changing World, vol. II, Guangzhou: Guangdong People's Publishing House, China, 2008, p. 833.
[19] C.J. Li, X.X. Zhang, F. Li, Z.B. Nan, C.L. Schardl, Disease and pests resistance of endophyte infected and non-infected drunken horse grass, in: A. Popay, E.R. Thom (Eds.), Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses, New Zealand Grassland Association, Dunedin, New Zealand, 2007, pp. 111–114.
[20] D.E. Zaurov, S. Bonos, J.A. Murphy, M. Richardson, F.C. Belanger, Endophyte infection can contribute to aluminum tolerance in fine fescues, Crop Sci. 41 (2001) 1981–1984.
[21] M. Fabien, V. Nathalle, H. Adnan, C. Alain, S. Huguette, Endophytic *Neotyphodium lolii* induced tolerance to Zn stress in *Lolium perenne*, Physiol. Plant. 113 (2001) 557–563.
[22] Z.F. Wang, C.J. Li, W.J. Jin, Z.B. Nan, Effect of *Neotyphodium* endophyte infection on salt tolerance of *Hordeum brevisubulatum* (Trin.) Link, Acta Agrestia. Sinica 1 (2009) 88–92.
[23] C.J. Li, Biological and ecological characteristics of *Achnatherum inebrians*/*Neotyphodium* endophyte symbiont, PhD Dissertation, Lanzhou University, China, 2005.
[24] R. Gucci, L. Lombardini, M. Tattini, Analysis of leaf water relations of two olive (*Olea europaea*) cultivars differing in tolerance to salinity, Tree Physiol. 17 (1997) 13–21.
[25] Y.P. Zhang, Z.B. Nan, Growth and Anti-Oxidative systems changes in *Elymus dahuricus* is affected by *Neotyphodium* endophyte under contrasting water availability, J. Agron. Crop Sci. 193 (2007) 377–386.
[26] A. Clairborne, Catalase activity, in: R.A. Greenwald (Ed.), Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 283–284.
[27] W.F. Beyer, I. Fridovich, Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions, Anal. Biochem. 161 (1987) 559–566.
[28] B. Chance, A.C. Maehly, Assay of catalase and peroxidases, Methods Enzymol. 11 (1955) 764–775.
[29] A.S. Gupta, P. Robert, A. Webb, H. Scott, R.D. Allen, Overexpression of superoxide dismutase protects plants from oxidative stress, Plant Physiol. 103 (1993) 1067–1073.
[30] M.M. Bradford, A rapid and sensitive technique to determine protein concentrations, Anal. Biochem. 72 (1976) 248–254.
[31] V. Velikova, I. Yordanov, A. Edreva, Oxidative stress and some antioxidant system in acid rain-treated bean plants: protective role of exogenous polyamines, Plant Sci. 151 (2000) 59–66.
[32] H.S. Li, Principle and Techniques of Botanic, Chemical and Physiological Experiments, Senior Education Press, Beijing, 2000, pp. 164–169, 194–197.
[33] C.J. Li, F. Li, X.Y. Gou, Z.B. Nan, Effects of abiotic stresses on *Achnatherum inebrians* by symbiotic endophyte of *Neotyphodium gansuense*, in: Organizing Commettee 2008 IGC/IRC Conference (Ed.), Multifunctional Grasslands in a Changing World, vol. II. Guangzhou: Guangdong People's Publishing House, China, 2008, p. 819.
[34] T. Melis, E. Selim, O. Faruk, H. Soren, C. Ismail, Antioxidant defense system and cadmium uptake in barley genotypes differing in cadmium tolerance, J. Trace Elem. Exp. Med. 20 (2006) 181–189.
[35] M.G. Hunt, S. Rasmussen, P.C.D. Newton, A.J. Parsons, J.A. Newman, Near-term impacts of elevated CO_2, nitrogen and fungal endophyte infection on *Lolium perenne* L. growth, chemical composition and alkaloid production, Plant Cell Environ. 28 (2005) 1345–1354.
[36] J.A. Newman, M.L. Abner, R.G. Dado, D.J. Gibson, A. Brookings, A.J. Parson, Effects of elevated CO_2, nitrogen and fungal endophyte-infection on tall fescue: growth, photosynthesis, chemical composition and digestibility, Glob. Change Biol. 9 (2003) 425–437.
[37] H.W. Elbersen, C.P. West, Growth and water relations of field-grown tall fescue as influenced by drought and endophyte, Grass Forage Sci. 51 (1996) 333–342.
[38] C.M. Bowler, V. Montagu, D. Inze, Superoxide dismutase and stress tolerance, Annu. Rev. Plant Physiol. 43 (1992) 83–116.
[39] G.A. Abernethy, M.T. McManus, Biochemical response to an imposed water deficit in mature leaf tissue of *Festuca arundinacea*, Environ. Exp. Bot. 40 (1998) 17–28.
[40] M. Bonnet, O. Camares, P. Veisseire, Effects of zinc and influence of *Acremonium lolii* on growth parameters, chlorophyll a fluorescence and antioxidant enzyme activities of ryegrass (*Lolium perenne L.cv Apollo*), J. Exp. Bot. 51 (2000) 945–953.
[41] C.M. Yeh, P.S. Chien, H.J. Huang, Distinct signalling pathways for induction of MAP kinase activities by cadmium and copper in rice roots, J. Exp. Bot. 58 (2007) 659–671.
[42] W. Maksymiec, Z. Krupa, The effects of short-term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*, Environ. Exp. Bot. 57 (2006) 187–194.
[43] A. Wahid, M. Perveen, S. Gelani, S.M.A. Basra, Pretreatment of seed with H_2O_2 improves salt tolerance of wheat seedlings by alleviation of oxidative damage and expression of stress proteins, J. Plant. Physiol. 164 (2007) 283–294.
[44] J. Doussiere, J. Gaillard, P.V. Vignais, The heme component of the neutrophil NADPH oxidase complex is a target for aryliodonium compounds, Biochemistry 38 (1999) 3694–3703.

[45] E. Olmos, J.R. Martinez-Solano, A. Piqueras, E. Hellin, Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line), J. Exp. Bot. 54 (2003) 291–301.
[46] M. Rodríguez-Serrano, M.C. Romero-Puertas, A. Zabalza, F.J. Corpas, M. Gómez, L.A. del Río, L.M. Sandalio, Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo, Plant Cell Environ. 29 (2006) 1532–1544.
[47] Y.T. Hsu, C.H. Kao, Toxicity in leaves of rice exposed to cadmium is due to hydrogen peroxide accumulation, Plant Soil 298 (2007) 231–241.
[48] C.M. Yeh, P.S. Chien, H.J. Huang, Distinct signalling pathways for induction of MAPkinase activities by cadmium and copper in rice roots, J. Exp. Bot. 58 (2007) 659–671.
[49] T.J. Buckhout, P.F. Bell, D.G. Luster, R.L. Chaney, Iron-stress induced redox activity in tomato (*Lycopersicum esculentum* Mill.). Is localized on the plasma membrane, Plant Physiol. 90 (1989) 151–156.