Article

The Promotion of *Festuca sinensis* under Heavy Metal Treatment Mediated by *Epichloë* Endophyte

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Abstract: To more clearly clarify the relationship between the *Epichloë* endophyte and its host, *F. sinensis*, the effects of *Epichloë* endophyte on *F. sinensis* performance under heavy metal treatment was investigated. The growth performance and physiology variations of *F. sinensis* with (E+) and without the endophyte (E−) were evaluated after they were subjected to Zn²⁺ and Cd²⁺ treatments. The results showed that heavy metal treatments had significant effects on plants, as the performance of plants under Zn²⁺ and Cd²⁺ treatments was significantly different with plants under control treatment ($p < 0.05$). Cd²⁺ treatments showed a hormesis effect, whereas Zn²⁺ did not. The endophyte increased host heavy metal stress tolerance by promoting host growth as the E+ plants had significantly higher plant height, tiller number, root length ($p < 0.05$). The endophyte also promoted ion uptake by the host and induced endogenous hormone production ($p < 0.05$). These results suggested that the *Epichloë* endophyte regulated host growth and physiology to improve association tolerance to environmental conditions. This study provides another example that the *Epichloë* endophyte can increase plant tolerance to metal stress.

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Keywords: alkaloids; Cd²⁺ treatment; *Epichloë sinensis*; hormone; host growth; ion absorbing; Zn²⁺ treatment

1. Introduction

Festuca sinensis Keng ex E.B. Alexeev, is a native cool-season perennial grass species distributed across the cold and semi-arid regions of China. This species, grazed by cattle and sheep, is widely utilised in grassland production on the Qinghai–Tibet plateau of China [1,2]. It is also important for grassland establishment, restoration of degraded grassland and ecological management [3]. *F. sinensis* is frequently infected with an asexual, symptomless *Epichloë* species [4–8]. This endophyte has been isolated and identified by morphology with colony, texture, conidia and conidiophore, and phylogene with house-keeping gene, which confirmed that the strain is new species name after *Epichloë sinensis* [9]. *Epichloë* endophytes interact mutualistically with their host plant, mainly by enhancing the fitness of the grass host and protection from both biotic and abiotic stresses [10,11]. Research to reveal the relationship between *F. sinensis* and *Epichloë* endophytes showed that associations between *F. sinensis* and endophytes produce alkaloids [12], and endophyte could increase *F. sinensis* seed germination and seedling growth [13], competition in mix-sowing grassland [14], enhance host cold-stress resistance [6,13], and improve host drought and waterlogged condition resistance [15]. However, the effects of the endophyte on host tolerance to other stress such as heavy metals and salt have not been clarified.

With the increasing industrialization of the global economy over the last century, heavy metals have been introduced into the environment and have caused extensive environmental problems [16]. Phytoremediation, the use of green plants to remove pollutants from the environment or to render them harmless, is an in situ, solar-powered remediation

technology [17]. This technology can be used to clean up and/or stabilize heavy metal contaminants, and has been considered to be the most promising technology due to its minimal site disturbance and low cost when compared with conventional remediation methods [18]. Microbe-assisted phytoremediation is a novel and promising concept. So far, numerous studies have demonstrated that microorganisms can enhance phytoremediation efficiently [19]. *Epichloë* endophytes can also increase the host stress tolerance of heavy metals such as cadmium (Cd^{2+}), aluminum (Al^{3+}), zinc (Zn^{2+}) and copper (Cu^{2+}) tolerance [20–23]. However, the relationship between *E. sinensis* endophyte and *F. sinensis* under heavy metal stress is unknown. The aims of this study were to investigate the performances of *F. sinensis*—endophyte interactions under heavy metal treatment, and to evaluate the possibility of employing the *F. sinensis*—endophyte association for phytoremediation in heavy metal polluted soils.

2. Materials and Methods

2.1. Plant Materials

F. sinensis seeds were collected from endophyte infected (E+) or endophyte free (E−) plants in summer, 2016 in experimental field blocks (104°39' E, 35°89' N, altitude 1653 m) at the College of Pastoral Agriculture Science and Technology (CPAST), Yuzhong campus of Lanzhou University [12]. The plants were grown from seed collected in Hongyuan, Sichuan (102°33' E, 32°48' N, altitude 3491 m) in 2013. Endophyte viability in seeds was assessed by aniline blue staining and microscopic examination [4]. After the seeds were treated with aniline blue solution, the sampled seed was marked as E+ if the longitudinally-orientated hyphae of the *Epichloë* endophyte were observed in the seed skin and aleuronic layer, and *vice versa*. After assessment, the seeds were stored in 4 °C until utilization. In August 2017, the well-filled, healthy-looking E+ and E− seeds were planted in plastic trays (30 cm × 25 cm × 8 cm) filled with 1.5 kg soil (commercial fine sandy soil, Lanzhou) which had been sterilized in an oven at 130 °C for 30 min. Five rows with 10 seeds were planted per tray at a depth of 1 cm. Two trays containing E+ and E− seeds were placed in a temperature-controlled greenhouse (18–24 °C) with 10 h of illumination per day in the Yuzhong campus of Lanzhou University. After plants had 3 tillers, endophyte viability in E+ and E− population seedlings were determined by microscopic examination of host leaf sheath pieces after they had been stained with aniline blue [5]. After the leaf sheaths were gently peeled from the tissue and treated with aniline blue solution, the sampled plants were marked as E+ if the longitudinally-orientated hyphae of the *Epichloë* endophyte were observed in the tissues, and *vice versa*. The seedlings germinated from E+ seeds with hyphae of the endophyte were marked as E+ and the seedlings germinated from E− seeds without hyphae were marked as E−.

2.2. Experimental Design

The marked seedlings were transplanted into round pots (upper diameter 15.5 cm × lower diameter 11.5 cm × height 14 cm) containing the same amount of media (sterilized commercial vermiculite and black soil in a *w/w* ratio 3:1) which fill the pot. Each pot had only one similar growth seeding and equal initial water treatment. After one-month stabilization with the same irrigation, three different treatments were established, which included control treatment (CK), Zn^{2+} treatment (Zn , 500 $\text{mg}\cdot\text{L}^{-1}$ ZnCl_2) and Cd^{2+} treatment (Cd , 100 $\text{mg}\cdot\text{L}^{-1}$ CdCl_2). Each treatment has 5 replicates which were randomly placed in greenhouse maintained at a constant condition (temperature: 25 ± 2 °C, humidity: $42 \pm 5\%$). During the experimental period, the plants under control treatment were supplied with 100 mL water every 3 days, Zn^{2+} and Cd^{2+} treatments were watered with 100 mL ZnCl_2 solution of 500 $\text{mg}\cdot\text{L}^{-1}$ and 100 mL CdCl_2 solution of 100 $\text{mg}\cdot\text{L}^{-1}$ at 1st and 14th day, respectively, and 100 mL water were also supplied with every 3 interval days.

2.3. Experimental Evaluations

2.3.1. Determination of Endogenous Phytohormones

After 28 days growth, 2 g of fresh leaves were collected from each plant for gibberellin (GA₃), indole-3-acetic acid (IAA), cytokinins (CTK) and abscisic acid (ABA) content tests using enzyme-linked immunosorbent assays, following the procedure of kits which included extraction with phosphate-buffered saline (pH 7.4), coating the corresponding antibody of each phytohormones, blocking and detection at 450 nm (Danshi biology, Shanghai, China) [24].

2.3.2. Plant Growth

The whole plants were destructively harvested from pots after 28 days growth, washed with distilled water and dried on a filter paper. The height, root length and tiller number of each plant were recorded. All harvested plants were separated into roots and shoots and their fresh weight was recorded. Dry weight was obtained after oven-drying at 60 °C until a constant weight was reached. The dry aboveground and underground parts from each treatment were weighed separately to determine total dry matter per plant. After weighting, the plant materials were ground twice using a mixer mill (Retch 400MM, German) at 30 Hz for 2 min for analysis of alkaloid, Zn²⁺ and Cd²⁺ ion contents.

2.3.3. Measurements of Zn²⁺ and Cd²⁺ Ions Concentrations

Zn²⁺ and Cd²⁺ concentrations in plants were analysed using atomic absorption spectrometry (M6AA system, Thermo, Waltham, MA, USA) at 213.9 and 228.8 nm, respectively, after mineralization in mixture of acids (HCl-HNO₃-HF-HClO₄) [25,26].

The translocation factor (TF) is the concentration ratio of metal acclimatised in the shoot to that present in the root.

$$TF = C_{\text{shoot}} / C_{\text{root}}.$$

where C_{shoot} (mg·kg^{−1}) and C_{root} (mg·kg^{−1}) represent the metal concentration in the shoot and root, respectively [27].

2.3.4. Measurements of Alkaloid Concentrations

Concentrations of peramine and lolitrems B were measured using high performance liquid chromatography (HPLC) [28,29].

2.4. Statistical Analyses

All averages and standard errors of the difference (SE) of measurements were recorded in Excel software, and statistical analysis was performed using SPSS software (version 18.0, Chicago, IL, USA). After a Kolmogorov-Smirnov test for normality and one-way ANOVA for descriptive and test homogeneity of variance, a two-way ANOVA at the 95% confidence level was used to estimate the effects of endophyte and heavy metal treatments on host plants. A repeated-measures ANOVA with Fisher's least significant differences (LSD) test was used to determine whether differences between means were statistically significant.

3. Results

3.1. Plant Growth

Both heavy metal treatments and endophytes had significant effects on plant growth (Figures 1–3) and plant physiology (Figures 4–7).

The plant height (Figures 1A and 2) and tiller numbers (Figures 1B and 2) of the E+ plants were significantly higher ($p < 0.05$) than those of E− plants under two heavy metal (Zn²⁺ and Cd²⁺) treatments and control (except for tiller numbers). The heavy metal treatments had significant effects on plant height (Figure 1A). For both the E+ and E− plants, the plant height was highest under Zn²⁺ treatment and lowest under Cd²⁺ treatment

($p < 0.05$). However, heavy metal treatments had no significant effects on tiller numbers for both E+ and E− plants (Figure 1B).

The root length of E+ plants was significantly higher ($p < 0.05$) than that of E− plants under control and Zn²⁺ treatments (Figure 3A). Heavy metal treatments only had significant effects on the root length of the E+ plants as the E+ plants had significantly longer root length under control and Zn²⁺ treatments than that under Cd²⁺ treatment. The plant biomass (Figure 3B) of E+ plants was significantly higher ($p < 0.05$) than those of E− plants under Zn²⁺ treatment. For both the E+ and E− plants, the plant biomass was highest under Zn²⁺ treatment and lowest under Cd²⁺ treatment ($p < 0.05$).

Figure 1. Effect of *Epichloë* endophyte and Cd²⁺ and Zn²⁺ treatments on plant height (A) and tiller number (B) of *F. sinensis*. Note: Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

Figure 2. Photos showing comparative phenotypes of E+ and E− plants under different treatments after harvesting.

Figure 3. Effects of *Epichloë* endophyte and Cd²⁺ and Zn²⁺ treatments on plant root length (A) and biomass (B) of *F. sinensis*. Note: Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

3.2. Cd^{2+} and Zn^{2+} Ion Concentration

Both the aboveground (Figure 4A) and underground (Figure 4B) Cd^{2+} ion concentrations were significantly higher ($p < 0.05$) in E+ plants than those in E− plants under Cd^{2+} treatment. For both E+ and E− plants, aboveground and underground Cd^{2+} ion concentrations were significantly higher ($p < 0.05$) under Cd^{2+} treatment than those under control and Zn^{2+} treatments.

Figure 4. Effect of *Epichloë* endophyte and Cd^{2+} and Zn^{2+} treatments on the aboveground and underground concentration of Cd^{2+} and Zn^{2+} ions in *F. sinensis*; (A) aboveground Cd^{2+} ions, (B) underground Cd^{2+} ions, (C) aboveground Zn^{2+} ions, (D) underground Zn^{2+} ions. Note: Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

The aboveground Zn^{2+} ion concentration (Figure 4C) of E+ plants was significantly higher ($p < 0.05$) than that of E− plants only under Zn^{2+} treatment. For both E+ and E− plants, aboveground Zn^{2+} ion concentration were significantly higher under Zn^{2+} treatment than that under the control and Cd^{2+} treatments. The underground Zn^{2+} ion concentrations (Figure 4D) of E+ plants were significantly higher ($p < 0.05$) than that of E− plants under control and Zn^{2+} treatments. For E+ plant, the underground Zn^{2+} ion concentrations were highest under Zn^{2+} treatment and lowest under Cd^{2+} treatment ($p < 0.05$). For E− plants, the underground Zn^{2+} ion concentrations were significant higher under Zn^{2+} treatment than under control and Cd^{2+} treatments. There was no significant difference in the underground Zn^{2+} ion concentrations between the control and Cd^{2+} treatments.

The TF values of the Zn^{2+} and Cd^{2+} ions ranged from 0.56–1.16 (Figure 5A,B). The TF value of the Zn^{2+} ions (Figure 5A) of E+ plants were significantly higher ($p < 0.05$) than those of E− plants under Cd^{2+} treatment, whereas they were significantly lower ($p < 0.05$) than those of E− plants under the control treatment. For E+ plants, the TF values of the Zn^{2+} ions were highest under Cd^{2+} treatment and lowest under the control treatment ($p < 0.05$). For E− plants, the TF values of the Zn^{2+} ions were significantly higher under control treatment than that under the Zn^{2+} and Cd^{2+} treatments ($p < 0.05$). The TF values of the Cd^{2+} ions (Figure 5B) of the E+ plants were significantly higher ($p < 0.05$) than that of E− plants only under Cd^{2+} treatment. For the E+ plant, the TF value of the Cd^{2+} ions were highest under Cd^{2+} treatment ($p < 0.05$). For the E− plant, there was no significant difference in the TF value of the Cd^{2+} ions between these three treatments ($p < 0.05$).

Figure 5. Effect of *Epichloë* endophytes and Cd²⁺ and Zn²⁺ treatments on TF values of *F. sinensis*; (A) TF values of Zn²⁺ ions; (B) TF values of Cd²⁺ ions. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

3.3. Plant Hormone Concentrations

GA₃ concentrations (Figure 6A) in E+ plants were consistently higher ($p < 0.05$) than those in E- plants for all 3 treatments. For E+ plants, GA₃ concentrations were highest under Zn²⁺ treatment and lowest under Cd²⁺ treatment ($p < 0.05$). However, for E- plants, GA₃ concentrations were highest under control treatment and lowest under Cd²⁺ treatment ($p < 0.05$). The CTK concentrations (Figure 6B) in E+ plants were consistently higher ($p < 0.05$) than those in E- plants for all 3 treatments. For both E+ and E- plants, CTK concentrations were highest under Zn²⁺ treatment and lowest under Cd²⁺ treatment ($p < 0.05$). The IAA concentrations (Figure 6C) in E+ plants were significantly higher ($p < 0.05$) than those in E- plants for all 3 treatments. For both E+ and E- plants, IAA concentrations under control and Zn²⁺ treatments were significantly higher ($p < 0.05$) than those under Cd²⁺ treatment. The ABA concentrations (Figure 6D) in E- plants were significantly higher ($p < 0.05$) than those in E+ plants under Zn²⁺ treatment. For both E+ and E- plants, ABA concentrations were highest under Cd²⁺ treatment and lowest under Zn²⁺ treatment ($p < 0.05$).

Figure 6. Effect of *Epichloë* endophytes and Cd²⁺ and Zn²⁺ treatments on the concentration of GA₃ (A), CTK (B), IAA (C) and ABA (D) in *F. sinensis*. Note: Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

3.4. Alkaloids

E+ plants produced both tested alkaloids: peramine and lolitrem B, while E− plants did not produce any alkaloids (Figure 7). The heavy metal treatments only had significant effects on lolitrem B concentrations. There was no significant difference in peramine concentrations among these 3 treatments. The lolitrem B concentrations under Cd²⁺ treatment were significantly higher than those under control and Zn²⁺ treatments.

Figure 7. Peramine and lolitrem B concentrations of *F. sinensis* E+ plants under Zn²⁺ and Cd²⁺ treatments. Note: The left is the alkaloid peak. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

4. Discussion

Excessive Zn²⁺ in soil will cause heavy metal pollution and reduce plant growth, although it is a necessary trace element for plant growth [30]. A previous study has shown that 300 mg/L Zn²⁺ in soil inhibited tillering and leaf extension and reduced biomass of *Achnatherum sibiricum* [2]. Another study showed that 20 mg/L Zn²⁺ promoted *F. arundinacea* seed germination and biomass accumulation, whereas 50 mg/L Zn²⁺ inhibited seed germination [31]. However, our study showed that plant height and biomass of *F. sinensis* under 500 mg/L Zn²⁺ treatment significantly increased ($p < 0.05$) compared with control. The reason why this Zn²⁺ dosage did not show a hormesis effect in plants needs further clarification by a deeper characterization of the used soil such as organic matter content, soil pH and the actual bioavailability of zinc. Cd²⁺ is one of the heavy metals that is most toxic to plants. It disturbs plant physiological processes, including photosynthesis, respiration and nutrient element absorption, and seriously inhibits plant growth and development [32]. Cd²⁺ inhibited the germination and growth of *A. inebrians*, *Elymus dahuricus* and *Hordeum brevisubulatum*, leading to leaf yellowing, radicle browning and biomass reduction [33,34]. Cd²⁺ has a sustained inhibitory impact on seed germination and seedling growth of *A. sibiricum* [24]. The present study found that 100 mg/L Cd²⁺ inhibited plant height and biomass accumulation, which were consistent with the findings of other studies that showed Cd²⁺ usually inhibited plant growth.

In the present study, *Epichloë* endophytes significantly increased plant height, tiller number and biomass of *F. sinensis* under Zn²⁺ treatment, and significantly increased tiller number and root growth under Cd²⁺ treatment. Similar results were also found from previous studies which suggested that endophytes increased host tolerance to heavy metals and alleviated the toxicity. Bonnet et al. also found that endophytes increased perennial ryegrass tolerance to Zn²⁺ with increased aboveground biomass [21]. Endophytes promoted the performance of *Lolium perenne*, *F. arundinacea* and *A. sibiricum* tillering under Cd²⁺ treatment [2,22]. Endophytes can alleviate the toxicity of Cd²⁺ to *F. arundinacea* and *F. pratensis* as E+ plants have more biomass than E− plants. *Epichloë* endophytes can also increase host heavy metal stress tolerances, such as Cd²⁺, Al³⁺, Zn²⁺ and Cu²⁺ [20–22].

In the present study, endophyte improved the uptake of Zn²⁺ and Cd²⁺ ions in plants which suggests that endophytes may not reduce the toxicity condition of host plants. Especially for the TF value of Zn²⁺ and Cd²⁺ ions under Cd²⁺ treatment, the values of E+ plants were over 1 and also were significantly higher than E− plants, which showed

that Cd^{2+} ion translocation from roots to shoots remained very high. This was consistent with the higher concentrations of Cd^{2+} ions in aboveground plants. The changes of ion absorption and distribution may have relationship with root exudates. Previous studies showed that endophytes improved the phenolic contents of *F. arundinacea* and *A. sibiricum* which reduced the toxicity of heavy metals [35–37]. Phenolics in root exudates of *F. arundinacea* could chelate with some heavy metal ions, which reduced heavy metal activity and toxicity [38]. However, endophytes in the present study did not show these effects, and the ion absorption in plants need more explanation with other physiological measurements.

Heavy metal stress can disturb plant physiological processes, including photosynthesis, photoelectronic transfer and mineral nutrition absorption [39]. The response of hosts to abiotic stress such as heavy metals is very complicated. Endogenous hormones variation is one of the direct responses. Plant endogenous hormones are organic substances that regulate plant growth and development, which may be part of a signal-transduction pathway, and stimulate signal reactions for stress responses [40,41]. Studies have revealed that endogenous regulations (e.g., biosynthesis, transport, redistribution, and conjugation of plant hormones) play a crucial role during the acclimation process against stress [42,43]. Exogenous application of plant hormones has also been reported to enhance stress tolerance in plants affected by heavy metals [44–46]. In the present study, the four tested hormone (GA_3 , CTK, IAA and ABA) contents had significant variations under heavy metal stress. Zn^{2+} treatment increased the contents of GA_3 and CTK and reduced the contents of ABA. Cd^{2+} treatment reduced the contents GA_3 and IAA. Previous studies also revealed that Cd^{2+} treatment reduced the contents of IAA, ethylene and GA_3 in *Oryza sativa*, which suggests that Cd^{2+} stress disturbs the biosynthesis of endogenous hormones [47]. These changes of endogenous hormones confirmed that the plants utilize hormones during stress response. *Epichloë* endophytes also have significant effects on endogenous hormones in plants and increased GA_3 , CTK and IAA contents and reduced ABA contents. These results were consistent with the previous studies that demonstrated *Epichloë* endophytes change hormones to improve host stress tolerance [10,11,48]. Bunyard and McInnis reported that E+ tall fescue plants produced significantly more ABA in response to drought stress than did E– plants [49]. Some glasshouse-based research has indicated a similar endophyteE–enhancement of ABA concentration in tall fescue leaf tissue in response to drought [50]. Similar results were also observed in some Chinese native grasses like *A. inebrians* and *F. sinensis*. E+ Chinese wild rye (*Leymus chinensis*) plants have a higher SA content than E– plants, especially when they are exposed to *B. sorokiniana* and *C. lunata* [51]. Some endophytes have been reported to produce IAA and related indole compounds in culture [52,53]. The concentration increase in E+ plants may be also adjusted by the production of IAA and related compounds in planta by endophytes. Phytohormone production in planta may also induce the defense-related secondary metabolism in plants. However, far too little is known about the role that hormones may play in symbiosis and their direct effects on host fitness traits. The interaction of plants with heavy metal stresses are generally associated with immobilization in roots, ion balance, antioxidant defence, phytochelation, etc. More studies must be conducted to clarify these mechanisms.

The benefits that endophytes confer on plant health, and conversely, detrimental effects on animal health, are partially due to the production of biologically active alkaloids [54–56]. Two such important alkaloid classes are ergots and lolitrems (indole diterpenes), which cause neurotoxic effects on grazing and granivorous vertebrates. Two other classes of endophyte-derived alkaloids, peramine and lolines, are known to be highly active against invertebrates, yet have little or no activity against mammalian species [57]. Alkaloids may play roles in host biotic stress tolerance such as to pathogens and insects [58–61]. The contents of alkaloids varied with many factors, including endophytes, host genotypes and environmental conditions [48,62]. In the present study, the contents of lolitrem B increased under heavy metal stress, which suggest its association with stress tolerance.

Our study showed that endophytes can promote the growth and development of *F. sinensis* under heavy metal stress. The mechanism that endophytes employed in improving host heavy metals stress tolerance included increasing the content of growth hormones such as IAA and GA₃, reducing the content of ABA and adjusting the alkaloid contents. This study has hence provided more evidence about the *Epichloë* endophyte relationship with hosts, and extended the symbiosis research to more native species. These results are important for understanding of microbe-assisted phytoremediation. As the interaction between host–endophyte and abiotic stress tolerance is complicated, additional studies are being conducting to understand the mechanisms for endophyte-mediated heavy metal stress tolerance.

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