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Research Article

Epichloe endophyte modifies antioxidative defense and aquaporin genes expression in response to Ni contamination in *Lolium perenne*

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Abstract

The aim of this study was to determine the impacts of endophyte (E) inoculation on Nickel tolerance of the *Lolium prenne* by measuring the physiological and biochemical traits in two populations of ryegrass consisting both (E-) and (E+) plants cultivated under Ni stress. The plants were grown in a Ni polluted soil at 0, 30, 90 and 180 mg Ni kg $^{-1}$ using a factorial experiment based on a completely randomized design with three replications. The present research showed that the activity of antioxidant enzymes increased in Ni treated plants in contrast to the control plants. Whereas the activity of glutathione reductase enzyme decreased. Also, endophyte infection increased the activity of some antioxidant enzymes but decreased the activity of superoxide dismutase enzyme. Upregulation of aquaporin gene (*LpTIP1;1*) was shown in population 1 E+ plants at all treatments and 30 ppm Ni in E- plants, whereas, the expression of *LpTIP1;2* in population 1 E- plants increased and no significant difference was shown for E+ plants. After 3 months from treating plants, considerable reduction in shoot biomass of E+ and E- plants was observed in 180 ppm Ni and endophyte inoculation decreased the biomass of plants. The shoot water content of E- plants was greater than E+ counterparts. Also, a significant increase of Ni concentration of roots and shoots was observed under Ni stress and the Ni concentration of E+ plants was 88.7% and 12.7% greater in shoots and roots, respectively, than non-infected plants. Overall, results suggest that variation in response to Ni stress in E+ and E- ryegrass populations may aid survival of the grass under stress conditions.

Keywords: Aquaporin, Endophyte, *Epichloe festuca*, Nickel, Reygrass

Introduction

Cool-season perennial grasses create interrelation with numerous fungal endophytes. These fungi grow in the intercellular spaces of grass aboveground tissues, concentrating in leaf sheath and stem base without displaying clear indications (Rodriguez *et al*., 2009). Interrelation with leaf-placed *Epichloe* spp. fungal endophytes, distinguished as asymptomatic symbioses, results specially in a range of accommodations of endophyte-inoculated grasses to abiotic and biotic stresses (Malinowski and Belesky, 2019). In the major fodder grass of *Lolium perenne* that has been the topic of several researches of endophyte–grass associations, physiological responses of the host to endophyte inoculation probably depend on stress elements and host genotypes (Assuero *et al*., 2006; Raeisi-Vanani *et al*., 2020). Considering the fact that *L. perenne* is more widely distributed in grasslands, it is important to assess the capacity of endophyte fungi in improvement of defensive responses of host plants during abiotic and biotic stresses.

Heavy metal stress stimulates a number of physiological and biochemical responses, which occur frequently during water stress (Barcelo and Poschenrieder, 2008). In plants, higher levels of Nickel decrease the rate of metabolic pathways activities and decline water and nutrient uptake and transport (Gajewska *et al*., 2006). Heavy metals may result in water deficiency in plants that may in turn lead to decreased growth and productivity. Water channel proteins (aquaporins) control and facilitate the inactive transport of water molecules down a water potential gradient. Aquaporins belong to the large major intrinsic protein (MIP) family of transmembrane proteins, and two major classes of plasma membrane (PIPs) and tonoplast (TIPs) exist (Chaumont and Tyerman, 2014). Overexpression of *BjPIP1* in tobacco leads to the increased tolerance in Cd-stressed transgenic plants, suggesting the involvement of aquaporins in metal stress resistance (Zhang *et al*., 2008).

Many researchers have identified a number of genes involved in plant response and tolerance to heavy metal stress. They showed improved tolerance to the stress in transgenic plants by the overexpression of a certain gene. However, identifying candidate genes in plants

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responsive to Nickel is poorly studied. Epichloe endophytes may trigger the activity of many genes estimated to be up to one third of the host genes (Dupont *et al*., 2015). However, little is known or documented about the participation of aquaporin genes to the increased tolerance to Nickel in endophyte-infected plants. In this paper, we assayed the response to Ni stress by studying their genes expression levels of TIPs in two infected and non-infected populations of *Lolium perenne*.

Materials and methods

Plant materials: Two populations of ryegrass (*L. perenne*) were applied as plant materials. These two populations were collected from natural grasslands of Iran and had distinctive morphological characteristics regarding plant height and tillering ability. Meanwhile, these populations were selected because of their high infection rates with *Epichloe festucae* var. lolii. The removal of endophytic fungi from E+ plants was carried out by using a mixture of two Folicur and Propiconazole fungicides (Sabzalian and Mirlohi, 2010). E+ and E- clones were allowed to proliferate and produce new tillers in the field. Endophyte status in leaf sheaths of all new tillers was determined before the start and at the end of experiment. The existence or absence of the fungus in E+ and E− clones, respectively, were examined by staining plant leaf tissues with Rose Bengal as explained by Saha *et al*. (1988). The results revealed that the infection rates was kept 100% for E+ and 0% for E-. Also, endophyte mycelium was not detected in the roots of ryegrass. Then, fifteen tillers, of approximately equal size from newly emerged tillers of E+ and E- plants were selected and transplanted into culture pots (size: 20*15 cm) filled with four levels of Ni polluted soil in three replicates. Soil used for the experiment was obtained from Shervedan research station of Isfahan University of Technology, located in the Central District of Falavarjan County, Isfahan Province, Iran (32° 32′ 21″ N 51° 29′ 21″E.). The contaminated soil was made to contain Ni^{2+} (as $NiCl_2$-H_2O) at different concentrations: 0, 30, 90, and 180 mg kg^{-1} soil. The experiment was performed in a completely randomized design (CRD) with factorial combinations of the treatments under greenhouse conditions. The mean temperature was 25-35°C and the photoperiod was 16:8 light: dark in a greenhouse.

In both populations of *Lolium*, in concentration of 180 ppm Ni, endophyte-free seedlings became severely toxic and destroyed. After 1 month, effects of nickel, endophyte and population on activities of various antioxidant enzymes and expression of *Lptip1;1* and *Lptip1;2* genes were investigated in E+ and E- ryegrass. After 3 months, all tillers and leaves of the plants were collected, weighed to estimate fresh weight, and then dried after at least for 2d at 75°C. Then, the average dry weight of plants was written for all treatments and the water content was also measured.

Assays of antioxidant enzymes activities: One hundred mg of the fresh shoot tissues (from 60-days plantlets) were ground in 1.5 ml of PBS (50 mM, pH 7.8) containing 0.1 mM EDTA. After centrifuging at 12,000 g for 20 min at 4ºC, the upper aqueous phase was used as a source for crude enzyme activity test. Ascorbate peroxidase, guaiacol peroxidase, superoxide dismutase, glutathione reductase and glutathione S-transferase activities were assessed according to Nakano and Asada (1987), Lin and Kao (1999), Beyer and Fridovich (1987), Halliwell and Foyer (1978) and Carmagnol *et al*. (1981), respectively.

Mineral concentration: To prepare samples for Ni measurement, dried roots and shoots tissues were digested in the concentrated HNO_3 at 160°C. After cooling, extracts were made up to 25 ml final volume with 1 M HCl. The plant extracts were then analyzed for nickel (atomic absorption spectrophotometry (GBC 932 AB PLUS type) measurements (Reeves *et al*., 1996).

Quantitative real time PCR: Expression of *Lolium perenne* tonoplast proteins *LpTIP1;1* and *LpTIP1;2* (accession number FJ472348.1 and FJ472349.1) was evaluated by real time RT–PCR. Total RNA was extracted from the leaves using Qiazol (Qiagen, 79306). Synthesis of cDNA was carried out by Quantiscript reverse transcriptase (Qiagen, 205311). Real-time PCR experiment were performed using the Qiagen apparatus (Rotor-Gene, USA) with SYBR Premix Ex Taq (TaKaRa, RR081Q). Gene-specific primer pairs were as follows: Actin: 5-CGCCATCCAGGCTGTGCTTTC-3 and 5-GATGGTGTCAGCCATACCGTG-3; *LPTIP1;1*: 5-GCGGCAACATCAGCCTCCTCA-3 and 5-TCATGACGATCTCGAACACC-3; *LpTIP1;2*: 5-GCGGCAACATCACCCTCTTCC-3 and 5-TCATGACGATCTCCAGCACA-3. The $2^{\Delta\Delta Ct}$ method was employed to quantify the gene expression profile (Livak and Schmittgen, 2001).

Statistical analysis of data was performed based on a completely randomized factorial design using SAS (Version 8) statistical software. All experiments were performed at least in triplicate. Statistical differences between the mean values were compared using LSD test after analysis of variance (ANOVA). Results are reported as mean ± standard error (SE) and $P<0.05$ was considered for statistical significance.

Results

Regardless of endophyte condition and Ni concentration in the soil, population 1 of *Lolium* showed higher fresh weight in the shoots compared with population 2. Shoot fresh weight of plants in population 1 was significantly decreased under 180 ppm Ni stress in both E+ and E- plants compared with the control. E+ plants of population 1 had lower shoot fresh weight compared with their non-infected counterparts. In contrast to population 1, shoot fresh weight of plants in population 2 did not show significant differences in both E+ and E- plants compared with the control (Figure, 1).

The water content of *Lolium* shoots, as indicated by the increase in fresh weight/dry weight ratio, increased

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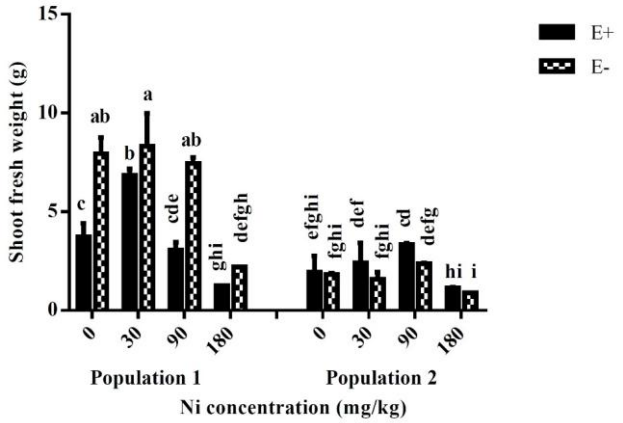

Figure 1. Influence of endophyte on shoot fresh weight of the two populations of *Lolium* exposed to soil contaminated with 4 levels of Nickel after 12 weeks. Bars represent standard error and different letters indicate significant differences by LSD test at P < 0.05, n = 3.

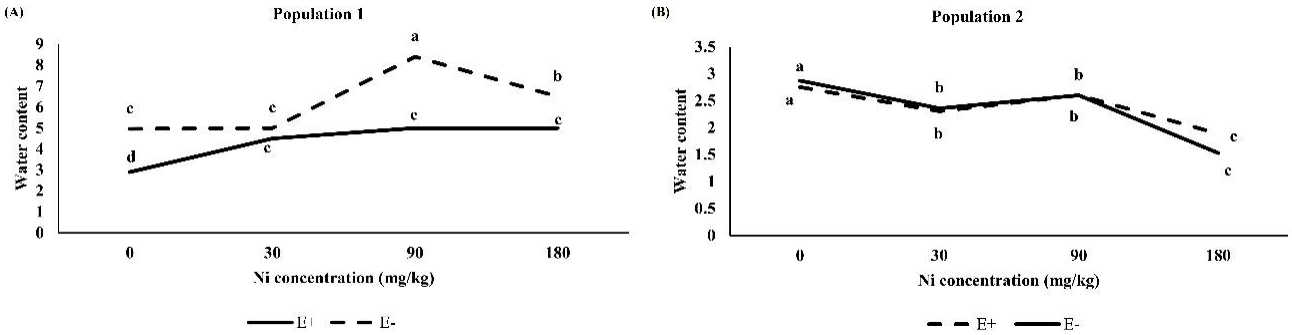

Figure 2. Influence of endophyte on water content of the population 1 (A) and population 2 (B) of *Lolium* exposed to soil contaminated with 4 levels of Nickel after 12 weeks.

significantly in both E- and E+ plants of population 1 when subjected to nickel stress. The water content of endophyte-free plants (E-) was 1.1, 1.7 and 1.3 folds higher than E+ counterparts at 30, 90 and 180 ppm Ni, respectively (Figure, 2A). In population 2, there was similar trend in water content between the endophyte-free and endophyte-infected plants in response to Ni stress (Figure, 2B).

Our results showed that in E+ plants of population 1, the activity of SOD enzyme significantly increased in all concentrations of Ni compared with the control E+. The activity of SOD in E- plants of population 1 exhibited no significant differences under Ni stress (in both populations of *Lolium*, in concentration of 180 ppm Ni, endophyte-free seedlings became severely damaged and destroyed). Conversely, SOD activity increased in 30 and 90 ppm Ni treatments in E- plants of population 2. In infected *Lolium* plants of the population 2, SOD activity was not significantly changed in all concentrations of Ni (Figure, 3A).

Under Ni stress, in the E+ plants, the activity of APX in the two populations was different. In population 1, the activity of this enzyme enhanced in concentrations of 30 and 90 ppm Ni, whereas in the population 2, the activity decreased. The activity of APX was only increased in shoots of E- plants in population 1 in 30 ppm Ni and in 30 and 90 ppm Ni treatment in population 2 (Figure, 3B).

Epichloe endophyte significantly affected the activity of GPX in E+ plants of population 1, but its activity did not show any change in E- plants of this population in all treatments. In population 2, GPX activity enhanced in 30 ppm Ni in E+ plants, but decreased in 90 and 180 ppm Ni stress, and there was no important alteration in GPX activity in E- plants under Ni stress (Figure, 3C). In population 1, activity of GR enzyme decreased in E+ and E- plants in all concentrations of Ni. Similarly, activity of GR enzyme decreased in E+ plants of population 2 in all treatments, and no significantly change was observed in E- plants in 30 and 90 ppm Ni. However, the activity of GR was higher in the shoots of E+ plants compared with the E- counterparts in both populations in all treatments (Figure, 3D). Also, the activity of GST increased in E+ plants in both populations in all treatments and higher activity of GST was found in the plants of population 1 compared with those of population 2 (Figure, 3E). In non-infected *Lolium* of population 1, the activity of GST decreased, while in population 2, GST activity increased in 30 and 90 ppm Ni.

According to the results shown in table 1, the concentration of Ni in shoots and roots of the two populations of *Lolium* (both E- and E+) was similar with the soil concentration of the metal. In the

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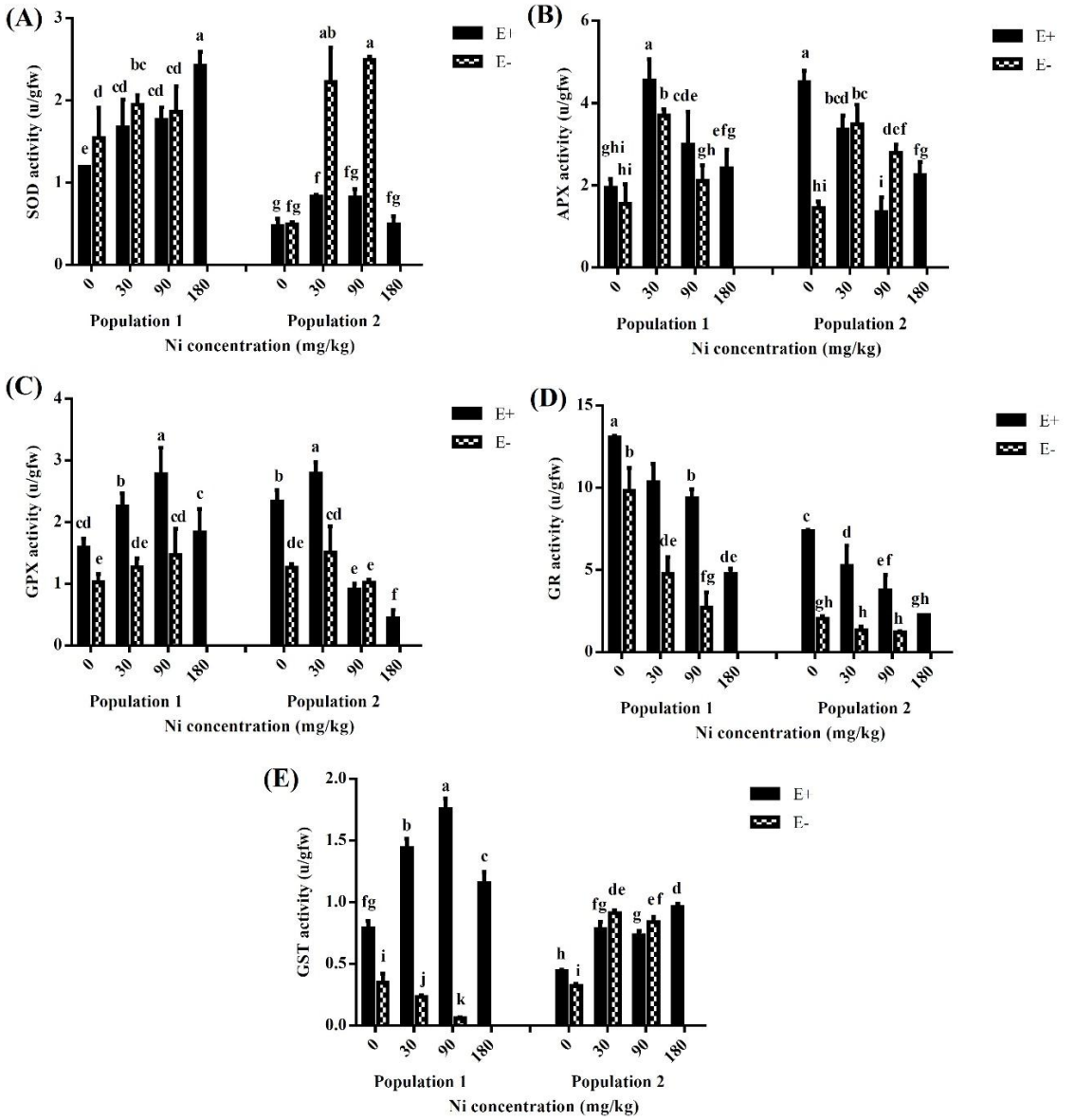

Figure 3. Influence of endophyte on antioxidant enzyme activities of SOD (A), APX (B), GPX (C), GR (D) and GST (E) of the two populations of *Lolium* exposed to soil contaminated with 4 levels of Nickel after 4 weeks. Bars represent standard error and different letters indicate significant differences by LSD test at P < 0.05, n = 3.

population 1, higher Ni contents was measured in the shoots and roots of E+ plants compared with E- plants in 90 and 180 ppm Ni treatment (Table 1). In contrast, in the population 2, the shoots of E- plants contained higher Ni concentrations than E+ counterparts when exposed to all concentrations of Ni. Whilst, in this population the roots of E+ plants contained higher Ni concentrations than E- counterparts.

The combinatorial effects of endophyte and Ni on *TIP* genes expression induction were investigated by real-time PCR analysis in population 1 (Figure 4). According to our previous study, the population 1 was more important than population 2 due to the production of more tillers and, also the absorption of more Ni in the shoots under Ni stress. Therefore, the population 1 was used for this part of the study. A significant increment of *TIP 1*;*1* expression was observed in E+ *Lolium* plants in all Ni treatments compared with the control. While the in *TIP* 1;1 the expression of E- plants increased only at Ni 30, but not in Ni 90 and Ni 180 treatments, compared with the control treatment (Figure, 4A).

Evaluation of *TIP 1*;*2* gene expression in E+ plants indicated that there was a significant enhancement of *TIP 1*;*2* expression in all Ni treatments compared with the control. While, there was no significant difference in *TIP 1*;*2* expression in E- plants in comparison with the control (Figure, 4B).

Discussion

Perception of the consequences of heavy metal stress in plants infected by endophyte fungi and the physiological processes involved in the enhanced tolerance of symbiotic plants to metal stress has been increased in recent years. However, there are still many

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Table 1. Nickel concentration in shoot and root of endophyte-infected and endophyte-free populations of *Lolium* under four Ni treatments. Values represent means ±SE of n = 3 replicates.

Treatment / Population	Ni shoot (ppm)		Ni root (ppm)	
	E+	E-	E+	E-
Population 1				
Control	23 ± 1^{i}	32.05 ± 1.16^{k}	27.29 ± 1.33^{k}	18.29 ± 0.36^{i}
Ni 30	47.4 ± 0.89^{hi}	44.1 ± 0.94^{hij}	82.24 ± 2^{i}	103.83 ± 4.46^{h}
Ni 90	109.91 ± 3.45^{d}	88.04 ± 1.51^{e}	$212.\ 6\pm4^{e}$	147.5 ± 7.11^{fg}
Ni 180	220.33 ± 16.86^{a}	167.63 ± 2.07^{b}	405.02 ± 2.46^{a}	380.61 ± 8.1^{b}
Population 2				
Control	9.32 ± 0.33^{m}	36.67 ± 1.16^{jk}	26.4 ± 0.36^{k}	23.52 ± 1.16^{ki}
Ni 30	40.12 ± 2.9^{ij}	48.31 ± 2.54^{h}	84.5 ± 1.84^{i}	65.22 ± 0.77^{i}
Ni 90	64.12 ± 1.12^{g}	80.83 ± 2.29^{ef}	151.27 ± 8.61^{f}	142.66 ± 1.17^{g}
Ni 180	75.04 ± 1.43^{f}	140.98 ± 1.97^{c}	262.78 ± 4.84^{c}	228.85 ± 3.01^{d}

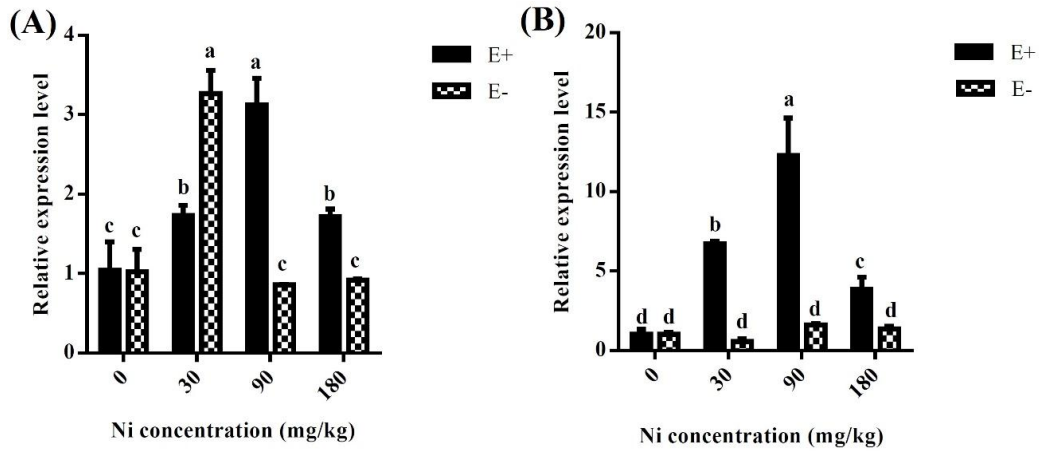

Figure 4. Influence of endophyte on genes expressions of *TIP 1;1* (A) and *TIP 1;2* (B) of the population 1 of *Lolium* exposed to soil contaminated with 4 levels of Nickel after 4 weeks. Bars represent standard error and different letters indicate significant differences by LSD test at P < 0.05, n = 3.

unknown aspects which must be elucidated, mainly at the molecular level. Symptoms of dryness and necrosis of Ni toxicity were observed on plant leaves at 180 ppm Ni after four weeks, regardless of endophyte infection. However, we found a significant increase in shoot biomass in plants exposed to 30 ppm Ni. In this treatment, tiller number significantly increased in comparison to the control whereas in other treatments, it decreased (Salehi *et al.*, 2014). Advantageous influence of Ni on growth has also been displayed for other plants (Arif *et al.*, 2016). Advantageous effects of endophyte infection on growth and water content host plants under metal toxicity were reviewed by Domka *et al.* (2019). Endophyte infection is known to mediate improvement of water uptake and nutrient uptake from the soil by mobilizing nutrients and making them available to the plant roots. The results of this study also showed that fresh weight of aerial parts of endophyte free plants in population 1 was conspicuously higher than that of the E+ plants, conversely, the fresh weight was higher in the existence of endophyte in the population 2, compared to the plants without endophyte. This might be probably related to the change in physiological features of the two populations. In comparison to population 2, endophyte-infected plants in population 1 indicated greater contents of chlorophyll a, carotenoid and phenolic compounds than endophyte-free plants (data not shown). Mesa *et al.* (2015) reported that endophyte inoculation improved plant photosynthetic traits and favored intrinsic water use efficiency, they suggested that the increase in growth can be attributed to the improvement in the photosynthetic carbon assimilation. Differences in shoot biomass between the two populations in response to endophyte infection suggest that endophyte may elicit different strategies for heavy metals stress tolerance in different ryegrass populations.

Ni concentrations increased significantly in plant shoots and roots in all treatments compared with the control. There may be mechanisms involved in metal tolerance, such as metal vacuolar localization, metal speciation and the production of metal chelators (Gasic and Korban, 2006). Regardless of endophyte infection, the observed Ni accumulation was higher in the roots compared to the shoots in all treatments. Also, concentration of Ni in shoots and roots was influenced by endophyte in both ryegrass populations, so that plants infected by endophyte demonstrated higher

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concentration of Ni in their shoots and roots than in non-infected ones. In population 1, in 180 mg Ni kg^{-1} soil, the Ni concentration of E+ plants were 88.7 % and 12.7 % higher in shoots and roots, respectively, compared with E- plants. Higher accumulation of the Ni in roots could be advantageous because of their capability to act as a place for depositing and deactivating of Ni and as a barrier for further transport of Ni to areal parts.

Considering the results related to enzymatic antioxidants in this study, irrespective of endophyte condition, two populations of *Lolium* demonstrated higher antioxidative potential in response to Ni stress. Between two populations, E+ plants of population 1 had higher induction on SOD, APX, GPX and GST enzyme activities under Ni stress. Endophyte inoculation simulates a more vigorous antioxidative system in host plants under stress and decreases damage of biomolecules at the cellular level (Dastogeer and Wylie, 2017). Increment in components of antioxidative system is usually related to enhance metal tolerance of host plants by various beneficial microorganisms (Ma *et al*., 2016). The effect of inoculation on the activity of antioxidant enzymes was confirmed in several other studies (Wang *et al*., 2016).

Additionally, the elevated SOD activity in shoots treated with high Ni concentration can be a defense response to Ni stress. Oxidative stress induced by metals is neutralized by the induction of superoxide dismutase and the production of antioxidant molecules (Gonzalez-Guerrero *et al*., 2009). It can be claimed that *Lolium* has a well capacity for fighting with ROSs that are produced under Ni toxicity through the activities of enzymes-coordinated induction implicated in their detoxification. APX activity remained unchanged in E+ plants of population 1 exposed to 90 and 180 ppm Ni, while in response to the treatment with 30 mg. kg^{-1} Ni, a significant increase (59.9 %) in the activity of this enzyme was found. In E- plants of population 2, APX activity increased in 30 and 90 ppm Ni (Fig. 3B). GPX activity of E+ plants increased in 30 and 90 ppm (in populations 1) and 30 ppm (in populations 2) Ni (Fig. 3C). Our results indicated that activation of APX and GPX enzyme activities in Ni 30 can play an essential function in the response of ryegrass E+ plants to Ni toxicity. It is suggested that H_2O_2 plays as a regularly intracellular signal for the activation of APX enzyme activity under abiotic stresses. Also, in plants activities of GPXs were considered as possible indicator for accessing sub-lethal, metal induced injury (Radotic *et al*., 2000).

Treatment of plants with higher Ni concentration caused in a reduction of the activity of GR in the shoots of E+ and E- plants of the two populations (Fig. 3D). Reduction in GR activity in plants under metal stress might be explained through 1) direct interaction with ions or ROS may result in the inactivation of enzyme, 2) the reduction in synthesis or impairment of protein assembly led to the inactivation of antioxidant enzyme (Rotilio *et al*., 1995). Our experiment demonstrates that prolonged treatment of ryegrass seedlings with Ni may result in the reduction of GR activity whereas GPX and APX activities enhanced in 30 ppm Ni treatment. This result indicated that GPX and APX have significant role in H_2O_2 scavenging in Ni-stressed E+ ryegrass plants. Also, a significant enhanced in GST activity was found in shoots of E+ plants of the two populations subjected to Ni stress (Fig. 3E). It has been proven that heavy metals including Ni induce peroxidation of membrane lipids. Therefore, it can be suggested that GST may be involved in the removal of toxic products of lipid peroxidation in plants under metal stress. In this study, GST seemed to be more important for detoxification of ROS in E+ plants of the two populations.

This result is interesting and was supported by the presence of endophyte in the leaves of the infected plants, which helped them to increase the threshold level of toxicity to Ni, as the increase in APX, GPX, GR and GST activities due to Ni treatment were greater in E+ plants than in E- ones. The results of this study demonstrated that the activities of SOD, GPX, GR and GST in the population 1 was higher than the population 2, while APX in the E+ plants of population 2 showed more activity than counterparts in population 1. However, by increasing the concentration of nickel in E+ plants, the activity of the enzyme was reduced.

Almost every plant process is affected directly or indirectly by the water supply, and water may be considered as a major factor in the regulation of plant growth. Therefore, many investigations on plant responses to environmental stresses pay considerable attention to water relations in plants. In plants, aquaporins responsive to several abiotic stimuli are located in cell plasma membrane and tonoplast. Numerous studies have been conducted regarding the effect of heavy metal stress on these proteins (Beaudette *et al*., 2007; Verdoucq *et al*., 2008; Zhang *et al*., 2008). In the present study, transcripts accumulations of *LpTIP1*;*1* and *LpTIP1*;*2* genes in shoots significantly enhanced in Ni treatments compared with the control. It clearly suggests that these two genes are transcriptionally controlled by Ni concentration, and Ni-sensitive transcriptional factors possibly mediate their regulation.

The results demonstrated that endophyte induced expression of *LpTIP1*;*1* and *LpTIP1*;*2* genes in shoots in all concentrations of Ni. A number of researchers have previously found the up-regulation of aquaporin in response to water stress in different plant species (Aroca *et al*., 2006; Yue *et al*., 2014). The transcript level of aquaporin is enhanced by *Trichoderma harzianum* colonization in rice (Pandey *et al*., 2016). Also, in E- plants, up-regulaion of *LpTIP1*;*1* gene was only observed in 30 ppm Ni treatment. In endophyte-free plants of *Lolium*, Ni-induced upregulation of this gene resulted in increased stress tolerance only at 30 ppm Ni. Results suggest that the induction of *LpTIP1*;*2* gene

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expression is unlikely to play a significant role in regulating aquaporin biosynthesis in E+ plants.

The plant cells constitutively expressing some of AQPs whilst the expression of others is controlled by various factors such as abscisic acid, gibberellic acid, developmental phase, salinity and drought (Vera-Estrella *et al*., 2004). It was recorded that ABA triggers the upregulation of transcription factors that control expression of aquaporins of the PIP subfamily (Shinozaki *et al*., 1998) and many studies reported the increment of PIPs mRNA following the ABA application (Zhu *et al*., 2005; Beaudette *et al*., 2007). However, the impacts of ABA on TIP aquaporin gene expression are not totally known. Based on De Battista *et al*. (1990) report, plant hormone ABA is known to be synthesized by endophytic fungus species in tall fescue, and there are several reports for upregulation of aquaporin by ABA. Therefore, increased expression of aquaporin gene in E+ plants may be a consequent of increased ABA hormone synthesis in these plants.

The different expression patterns between E+ and E- plants may reflect their distinctive necessity for water absorption and transfer in several tissues in response to stress. Also, proteins translated from *LpTIP1.2* like *LpTIP1.1* in tonoplast are involved in water transport. As pointed out, increased expression of *LpTIP1.2* in E- plants is correlated with the increased soil Ni concentration, which shows the need for more proteins translated by this gene, to be established in tonoplast under Ni stress. It is expected that the water content of tissues in E- plants be relatively higher than E+ plants, since E- plants have established more *LpTIP1.2* and *LpTIP1.1* translated proteins in tonoplast.

The results shows that the difference between fresh and dry weights of aerial parts was higher in E- plants in 90 and 180 ppm Ni compared to E+ counterparts. Therefore, in endophyte free plants of ryegrass, enhanced water transport through aquaporins in shoots are probably required, in favor of maintaining a appropriate water status under Ni stress. In general, the present study confirms the important role of *LpTIP1*;*1* and *LpTIP1*;*2* genes in the enhancement of water content of E- plants of perennial ryegrass and thereby alleviation of the damage caused by Ni.

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Disclosure statement

"The authors declare no conflicts of interest"

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