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Original article

Neotyphodium endophytes may increase tolerance to Ni in tall fescue

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ABSTRACT

Soil pollution with heavy metals is an important environmental problem which affects human and food health. *Neotyphodium* endophytes are a group of fungi which have their entire life cycle within the aerial parts of many cool-season grasses without any negative symptoms, and increase host tolerance to biotic and abiotic stresses. In this study, four groups of endophyte–host combinations including two genotypes of tall fescue either infected or non-infected by endophyte (75B E+, 75B E−, 75C E+ and 75C E−) were cultivated by using 10 equal sized tillers in each plastic pot containing Ni contaminated soil at concentrations of 30, 90 and 180 mg Ni per kg in 3 replicates. Growth parameters including root and shoot dry weight, tiller number of plants and chlorophyll and carotenoid content of shoots were measured at the end of the experiment. Antioxidant enzymes (CAT, SOD, APX, GPX, GR and GST) activities, total content of phenolic compounds and Ni accumulation in the roots and shoots after 10 weeks of growth was also determined. Results demonstrated that endophyte infection had a benefit to the plant growth and Ni tolerance in genotype 75B by improving the antioxidative system and by a reduction of Ni accumulation in the shoots. In genotype 75C, in contrast, the E− plants showed more tolerance to Ni stress compared to the E+ counterparts. It was revealed that the effect of endophyte infection on *Festuca* plants as the host may be dependent on the host genotype and endophyte*host interaction.

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1. Introduction

The prevalent association between endophytic fungi of the family Clavicipitacea and grasses has motivated numerous investigations into the ecological importance of this symbiosis [11–13,40]. The relationship between endophytic fungi and grasses is thought to be a mutualistic symbiosis [7,64]. The endophyte gains shelter, nutrition and diffusion via host propagules and grants some advantageous traits to host plants [14,45,61,63]. It has been widely reported that endophyte infection may lead to increased biotic and abiotic stress resistance and hence provides plants with widespread adaptability [7,35,53,45].

The best-studied endophytes are *Neotyphodium lolii*, colonizing perennial ryegrass (*Lolium perenne* L.), and *Neotyphodium coenophialum*, which colonizes tall fescue (*Festuca arundinacea* Schreb.). Tall fescue as a broadly cultivated grass found in temperate to cool zones worldwide has perennial habit, deep root system, wide range of pH tolerance (4.7–9.5) and an ability to grow in many soil types. It has received widespread consideration to be planted for soil erosion control and forage and turf grass production [15]. Although the effects of *Neotyphodium* endophytic fungi on tall fescue growth and development have been extensively studied [39,34,35,45,47,48], the role of *Neotyphodium* endophyte in metal stress resistance needs more scrutiny. Some recent studies have represented that endophyte infection may impress (modify) metal uptake and improve metal tolerance in host plants [56,67,73].

Nickel (Ni), is an essential metal for plants [22], and plays important roles in metabolism but also could become toxic at excess concentrations and therefore may be considered as an environmental pollutants often associated with serpentine soils [37]. Due to the increased industrial uses, today's Ni toxicity is a particular concern. Chlorosis, necrosis, inhibition of growth and wilting are the common symptoms of nickel toxicity in plants reported in the literature [43,52].

Plants subjected to high concentrations of nickel generate reactive oxygen species (ROS) such as $O_2^{\bullet-}$, $O^{\bullet}$ and $OH^{\bullet}$ [54] which by their over-production, expose plants to oxidative damage [4]. Antioxidative defense system in plant cells involving enzymatic and non-enzymatic antioxidants is also affected by ROS [30]. In response to ROS production, plants activate their antioxidative system to scavenge for excess ROS to avoid oxidative damage [70]. Superoxide dismutase (SOD) constitutes the first line of defense

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against ROS and converts O_2^- radicals to H_2O_2 [21]. Produced H_2O_2 then is deactivated by catalase [27] and peroxidases involving ascorbate peroxidase (APX) [70] and guaiacol peroxidase (GPX) [3]. Glutathione-S-transferase (GST) catalyzes the addition of reduced glutathione (GSH) to the substances having an electrophilic functional group [27]. The changes in the latest enzyme activity have been reported in plants under both biotic and abiotic stresses [24,58,18,27], however, there is not much information on the role of GST in heavy metal tolerance in plants [20]. Glutathione reductase (GR) is a flavo-protein oxidoreductase which plays a critical role in the defense system against ROS. This enzyme sustains the reduced status of GSH, a molecule involved in many metabolic regulatory and antioxidative processes. Both GR and GSH play a crucial role in determining the tolerance of a plant under various stresses [29].

Phenolics are one of the groups in the category of plant secondary metabolites characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. During heavy metal stresses, phenolic compounds can also show antioxidant function by chelating metals and scavenging molecular species of active oxygen [49]. In addition, they can repress the superoxide derived from the Fenton reaction which is believed to be the most important source of ROS [6,57]. An enhancement of the amount of phenolic compounds has been observed in plants under heavy metal stress conditions [71]. Malinowski et al. [75] reported that endophytic fungi enhance the release of phenolic-like compounds with Fe^{3+} reducing activity into the rhizosphere that may affect Fe uptake by tall fescue. However, there is no report of antioxidative activity of the endophyte-derived phenolics. Overall, what is known is that endophytic fungi can increase host tolerance to stresses, although the physiological functions affected are not specific to a single stress [23]. Therefore, there could be a crosstalk between mechanisms involved in endophyte-induced tolerance.

A substantial part of Iran is covered by serpentine soil particularly in western parts where Ni concentration is high [28] and cool season grasses normally and naturally grow. By reporting *Neotyphodium* endophyte infection in Iranian cool season grasses including tall fescue [59], one may hypothesize that a *Neotyphodium*–host combination may better tolerate the concentration of Ni in the soil or inside plant tissues. The main objectives of the present study was therefore to investigate: 1) the role of *Neotyphodium* endophyte on Ni tolerance of tall fescue correlated with antioxidant enzyme activity and phenolic compounds, and 2) the effect of *Neotyphodium* on Ni accumulation in tall fescue as a host plant.

2. Materials and methods

2.1. Plant materials

Two tall fescue genotypes (75B and 75C) infected by their natural fungal endophyte *N. coenophialum* (E+ plants) and their non-infected isolines (E− plants) were selected for this research. The E− plants were obtained by removing fungi from the E+ clones reported in the previous study [59]. Both E+ and E− plants were cloned for at least 3 generations before being used in this experiment. Endophyte status in leaf sheaths of all plants was confirmed by using the direct staining method [60] before the initiation and end of experimentation. Potting soil was provided from Lavark, research station of Isfahan University of Technology, Iran. Nickel (as $NiCl_2$) was added to the soils at concentrations of 0, 30, 90, and 180 mg kg^{-1} (control, Ni 30, Ni 90, and Ni 180). Then, 10 tillers of each plant, of approximately equal size, were cultivated in plastic pots (Size: 20*15 cm) filled with soil contaminated with 4 Ni levels in 3 replicates and were grown in a greenhouse (temperature 27 ± 3 °C, relative humidity 45 ± 8%, 12 ± 0.5 h day light). Pots containing E+ and E− versions of each plant genotype and each Ni treatment at three replications were placed in a random arrangement and their positions were changed weekly to prevent any systematic errors. Plants were watered as needed (about 4 times a week). After 10 weeks, plants were washed with deionized water. One gram of shoots was placed in an eppendorf tube, which was immediately placed into liquid nitrogen and stored at −80 °C until further analysis. The remained roots and shoots were also separated and oven-dried (65 °C) and then their weights were determined.

2.2. Determination of chlorophyll and carotenoids content

Total leaf chlorophyll and carotenoids were extracted by homogenizing 100 mg of leaf fresh weight in 10 ml 80% acetone. After centrifugation for 10 min at 4000 rpm, chlorophyll and carotenoids contents in supernatants were analyzed spectrophotometrically following the method of [5] and [41] at 470, 663 and 645 nm.

2.3. Preparations and assays of antioxidant enzymes activities

One hundred milligrams of fresh shoot tissue were homogenized in 1.5 ml of 50 mM sodium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. After centrifugation at 12,000 *g* for 20 min at 4 °C, the supernatant was used as a source for the crude enzyme activity test. All steps to obtain enzyme preparation were carried out at 4 °C.

2.4. Catalase assay

CAT activity was determined using the method described by Ref. [1]. The sample was added to a reaction solution containing 10 mM H_2O_2 in sodium phosphate buffer. CAT activity was measured by monitoring the decrease in absorbance at 240 nm and using an absorbance coefficient for H_2O_2 of 0.039 mM cm^{-1}. One unit of CAT gives a H_2O_2 decomposition rate of 1 μmol min^{-1} at 25 °C [1].

2.5. Ascorbate peroxidase assay

The activity of APX was determined using a procedure modified from that described previously by Ref. [51]. The reaction mixture contained 50 mM sodium phosphate buffer, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 μl of enzyme extract. Oxidation of ascorbic acid was followed as a decrease in absorbance at 290 nm. The enzyme activity was calculated using an absorbance coefficient for ascorbic acid of 2.6 mM cm^{-1}. One unit of APX oxidizes ascorbic acid at a rate of 1 μmol min^{-1} at 25 °C.

2.6. Guaiacol peroxidase assay

Peroxidase activity in shoot extracts was assayed using the method described by Ref. [42]. The assay mixture consisted of 9 mM guaiacol and 19 mM H_2O_2 in 50 mM phosphate buffer at 25 °C. The reaction product was measured at 470 nm. The extinction coefficient was 26.6 mM cm^{-1}. A unit of guaiacol peroxidase enzyme activity was defined as enzyme content which causes the formation of 1 μM tetraguaiacol in 1 min.

2.7. Superoxide dismutase assay

The activity of SOD was assayed as described by Ref. [8]. The reaction mixture was prepared by mixing 25 ml of 50 mM L^{-1} potassium phosphate buffer, pH 7.8, 35 mg of L-methionine

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