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Effects of *Epichloë gansuensis* on root-associated fungal communities of *Achnatherum inebrians* under different growth conditions

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ABSTRACT

This study was conducted to investigate how *Epichloë gansuensis* endophyte and soil disturbance affect root-associated fungi (RAF) of *Achnatherum inebrians* (drunken horse grass, DHG), using Illumina sequencing techniques. The rhizosphere soil of wild endophyte-infected (W-EI) DHG had significantly ($P < 0.05$) higher available phosphorous and potassium, total organic matter, ammonium and nitrate nitrogen than cultivated soil. In addition, the rhizosphere soil of endophyte-infected DHG had significantly ($P < 0.05$) lower pH and nitrate nitrogen, and higher available phosphorous, than endophyte-free DHG under cultivated conditions. The sequencing provided a total of 54,413 sequences and these were assigned into 190 operational taxonomic units (OTUs) with 97% similarity. Ascomycota was the most dominant phylum in roots of three DHG populations. W-EI DHG had significantly ($P < 0.05$) higher RAF diversity than cultivated endophyte-infected (C-EI) DHG. The presence of endophyte significantly ($P < 0.05$) decreased RAF diversity under cultivation. The principal component analysis (PCA) and sample similarity analysis results indicated that both endophyte and soil disturbance could bring changes to RAF community composition. The RDA results demonstrated the RAF of W-EI DHG were positively correlated with soil properties, and the RAF of cultivated DHG roots were negatively correlated with soil properties. This study demonstrated that both endophyte and soil disturbance resulted in changes to the RAF communities.

1. Introduction

Plant tissues form a wide variety of symbiotic associations with microorganisms and the nature of the associations range from parasitism to mutualism (Baker et al., 1997; Van der Heijden et al., 2008; Philippot et al., 2013; Averill et al., 2014). An example of a mutualistic association with aboveground tissue is provided by systemic, seed-borne fungal endophytes belonging to the genus *Epichloë* (Leuchtmann et al., 2014). This type of fungal endophyte is found in many cool-season grasses of the subfamily Pooideae and hyphae are present in all tissues except roots (Christensen et al., 2008). Vegetative tissues of all associations are symptomless and transmission of many species is entirely vertical in nature, in the seed of host plants (Schardl et al., 2004; Christensen et al., 2008). However, with some *Epichloë* spp., horizontal transmission can occur arising from the production of stromata in which the sexual stage is produced and from which ejected ascospores can penetrate stigmata of flowers on neighbouring plants and colonize developing seeds (Chung and Schardl, 1997). The most studied associations are those involving grasses of the genera *Lolium* and *Festuca*, as they have enhanced persistence and productivity in forage livestock farming (Kuldau and Bacon, 2008; Becker et al., 2016; Soto-Barajas et al., 2016).

Roots are colonized, both internally and externally, by a wide range of root-associated fungi (RAF) (Keim et al., 2014; Wehner et al., 2014). The most studied RAF are those that form mutualistic mycorrhizal associations (Van der Heijden et al., 2015). The most abundant mycorrhizal fungi are those that form vesicles and arbuscules in roots and are connected to the soil via long hyphae (Smith and Read, 2008). They belonging to the phylum

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Glomeromycota, and are named arbuscular mycorrhizal fungi (AMF) (Smith and Read, 2008; Bonfante and Genre, 2010). Another type of mutualistic mycorrhizal association is that in which the fungi form a hyphal sheath over root tips and hyphae also surround the cortical cells; these ectomycorrhizal associations are common with tree species and provide a range of benefits (Van der Heijden et al., 2015; Martin et al., 2016). Most of the fungi producing ectomycorrhizal associations are Basidiomycota and Ascomycota (Van der Heijden et al., 2015).

Many other fungi form associations with roots with the nature of the associations including some that are probably mutualistic, some pathogenic, and some that are saprotrophic and breakdown dead root tissue, while the role of many is unknown (Hamilton and Bauerle, 2012; Wehner et al., 2014; Aguilar-Trigueros and Rillig, 2016). One group that is being intensively studied is referred to as the dark septate endophytes (DSE) (Bonfim et al., 2016; Santos et al., 2016). As with the known mycorrhizal fungi, they produce characteristic inter- and intra-cellular structures in root cortical tissues and some reports indicated that the associations between some DSE fungi and host plants might benefit both partners (Santos et al., 2016).

Drunken horse grass (*Achnatherum inebrians*, DHG), is a perennial bunchgrass in northwestern China, and is widely distributed in the arid and semi-arid grasslands in Gansu, Xinjiang and Inner Mongolia (Shi, 1997; Li et al., 2004). In these grasslands nearly every DHG plant will be host to either of two *Epichloë* spp., *Epichloë gansuensis* (formerly *Neotyphodium gansuense*) (Li et al., 2004; Leuchtmann et al., 2014) and *Epichloë inebrians* (Moon et al., 2007; Chen et al., 2015). Studies into the functional impacts of an *Epichloë* sp. in DHG have shown that its presence improves tolerance to some biotic and abiotic stresses including drought (Li et al., 2008), low temperature (Chen et al., 2016), some fungal pathogens (Xia et al., 2015, 2016), heavy metals (Zhang et al., 2010) and insect pests (Zhang et al., 2012). The presence of alkaloids, including ergonovine and ergine, in *E. gansuensis*-infected DHG plants, are associated with livestock toxicosis and thus they are little grazed (Zhang et al., 2014). Recent research has shown that these toxic plants provide a protected nursery enabling the reestablishment of the original plant flora in degenerated grasslands, the result of overgrazing (Yao et al., 2015). However, trials have indicated that endophyte-free DHG could be utilized as animal feed (Liang et al., 2017).

The traditional way that the colonization of roots by fungi was determined involves the isolation of fungi from surface-sterilized roots and also examination of clarified stained roots (Sun and Tang, 2012; Dalpé and Séguin, 2013). Although these procedures have provided much understanding there are limitations to the extent of information that can be obtained regarding the range of fungi present (Vierheilig et al., 2005; Sun and Tang, 2012). In recent years, sequencing techniques have enabled the identification of large numbers of fungal species in plants and soils in a wide range of environment conditions (Lin et al., 2012; Wehner et al., 2014; Kazeeroni and Al-sadi, 2016; Zhou et al., 2016a).

Grasses commonly form symbiotic relationships with many fungi including foliar endophytes and RAF such as AMF and DSE (Vandegrift et al., 2015; Slaughter and McCulley, 2016). *Epichloë* spp. endophytes are present in seeds prior to germination and their growth is synchronized with that of the host (vertical transmission) (Christensen et al., 2008), whereas RAF colonized roots horizontally (Smith and Read, 2008; Van der Heijden et al., 2015). Additionally, all of these fungi acquire carbon from host grasses, with the foliar-confined *Epichloë* endophytes being located within the tissues where photosynthesis occurs (Mack and Rudgers, 2008), and thus the presence of plant-nutrient dependent foliar endophytic fungi may inhibit the colonization of roots by some fungal species. Some studies have indicated that *Epichloë* endophytes can have different (suppression, promotion and no) effects on AMF, while having no effect on DSE in roots (Chu-Chou et al., 1992; Larimer et al., 2012; Vandegrift et al., 2015; Slaughter and McCulley, 2016; Zhou et al., 2016b). In addition, the RAF communities were commonly influenced by specific environmental factors (Blaalid et al., 2012, 2014; Fujimura and Egger, 2012; Yu et al., 2013). In the present study, the RAF communities of cultivated and wild DHG plants either with or without the foliar endophyte were examined. Based on previous findings two hypotheses were considered in this study. The first is that the occurrence of endophyte may influence or alter the RAF communities, and the second is that soil disturbance may also alter the RAF communities.

2. Materials and methods

2.1. Study site description and sample collection

The experimental study site, established in 2011, is located at the College of Pastoral Agriculture Science and Technology, Yuzhong campus (104°39′ E, 35°89′ N, attitude 1653 m) of Lanzhou University (Xia et al., 2015, 2016). A field area of DHG plants (32 endophyte-infected (EI) plots and 32 endophyte-free (EF) plots; each plot: 3.65 m × 1.30 m, 3 lines and 8 rows) were established using seeds collected from 100 EI and 100 EF DHG plants, respectively, that had been assessed by microscopic examination for the presence or absence of characteristic hyphae of *E. gansuensis* (Xia et al., 2015). To confirm that the seeds that were collected from both categories of plants were actually either EI or EF as expected, leaf sheaths of seedlings grown from each seed lot were stained with aniline blue and microscopically examined for the presence of the hyphae characteristic of the endophyte (Li et al., 2004, 2008). These plots were then established and were regularly watered and weeded. Four EI and EF experimental plots were randomly selected for this study. For each plot, five individual DHG plants were selected randomly, root and soil samples were collected using a 20-cm soil auger from close to each plant, roots samples were removed from each of the root and soil samples using tweezers following the soil being sieved, and these were combined as either root or soil samples. Four root and soil samples were also obtained from wild DHG plants at a nearby area of natural grassland (10 m × 100 m), with each root sample including lengths of five separate roots. The root and soil samples were placed in an icebox and transported to the laboratory. The root samples were gently washed with tap water several times, rinsed with sterile water, and then dried with sterilized filter paper. The root samples were stored at −80 °C prior to DNA extraction. Each of the four DHG plants in the natural grassland from which roots were obtained was later examined and this confirmed that all were infected with *E. gansuensis.*

2.2. Soil properties

The soil samples were air-dried, passed through a 2-mm sieve, and stored at 4 °C prior to being analyzed for pH (1:2.5, soil/water), available phosphorus (AP) (Olsen et al., 1954), available potassium (AK) (Helmke and Sparks, 1996), and soil organic matter (SOC) (Nelson and Sommers, 1982). Total nitrogen (TN), total P (TP), ammonium-N (AN) and nitrate-N (NN) in the soil were measured using a continuous flow analyzer (FIAstar 5000 Analyzer), and the plant-available N content was calculated as the sum of the AN and NN (Zhao et al., 2014).

2.3. DNA extraction and PCR

From each root sample ($n = 12$), 100 mg segments were