Plant Soil (2020) 452:185–206
https://doi.org/10.1007/s11104-020-04556-7

REGULAR ARTICLE

Fungal Endophyte Improves Survival of *Lolium perenne* in Low Fertility Soils by Increasing Root Growth, Metabolic Activity and Absorption of Nutrients

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Received: 21 October 2019 / Accepted: 30 April 2020 / Published online: 23 May 2020

Abstract
Background and aims Low nutrient soil is a major limiting factor for normal growth and high yield of plants. *Epichloë* endophyte infection has been shown to increase host growth, nutrient uptake and balance. This study was done to determine the impact of *Epichloë* endophyte on growth, survival and elemental nutrient content of perennial ryegrass (*Lolium perenne*) under low fertility conditions.
Methods endophyte-infected and endophyte-free plants of *L. perenne* were grown without fertilization in a greenhouse environment. Plant survival rate, dry weight of leaves and roots, root metabolic activity and nutrient element (C, N, P, Na, K, Ca, Mg, Cu, Fe, Zn, Mn) contents were determined after 0 d, 45 d, 90 d, 135 d and 180 d.
Results The presence of *Epichloë* endophyte relieved the withering and yellowing of plants in the short term (0 to 90 d). The possible mechanisms by which *E. festucae* var. *lolii* infection enhances survival of plants includes improved root growth and metabolic activity (i.e., cellular metabolism), increased biomass production, and increased concentration of many nutrient elements in leaves and roots. The endophyte had positive direct and indirect effects on plants in multiple ways, including: increased organic carbon, total nitrogen, total phosphorus and Mn content in leaves, increased K content in leaves and roots, and improved root growth and activity.
Conclusions The presence of endophyte *E. festucae* var. *lolii* played a key role in improving the survival of *L. perenne* plants by increasing root growth and metabolic activity, promoting plant biomass and altering nutrient content.

Keywords *Epichloë festucae* var. *lolii* · *Lolium perenne* · Perennial ryegrass · Nutrient content · Plant survival · Plant growth · Root metabolic activity

Responsible Editor: Anna Maria Pirttila.

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Introduction

Fertile soil leads to high yield and quality crops, but many crops are often grown on poor soil or marginal lands where soil fertility is low (Vopravil et al., 2015), so substantial quantities of inorganic fertilizers must often be used to obtain high yields of crops (Bihari et al., 2018). The excess use of chemical fertilizer in agricultural production leads to soil acidification (Wang et al., 2017a), environmental pollution (Ahmed et al., 2017) and harms human health (Ibrahim et al., 2013; Niyokuri et al., 2013). Cultivation of plant varieties resistant to low fertility stress is one approach that avoids these negative issues (Wu et al., 2011).

Some plants that form symbiotic relationships with microbes have increased tolerance to low nutrients because the microorganisms promote nutrient absorption and increase nutrient utilization efficiency of the host (Tanveer et al., 2014; Verzeaux et al., 2017; White et al., 2018). Fungal endophytes are important plant symbiotic microbes, and these fungi infect their hosts without causing apparent symptoms of disease (Delaye et al., 2013). The most studied group of endophytic fungi is that of genus *Epichloë* (= *Neotyphodium*, [Ascomycota, Fam. Clavicipitaceae]) (Leuchtmann et al., 2014; Li et al., 2004; Shymanovich et al., 2017). *Epichloë* endophytes systemically colonize the intercellular spaces of aerial tissues in various cool season grasses (subfamily Poöideae) such as *Festuca arundinacea* (Zabalgogeazcoa et al., 2013). *Epichloë* hyphae colonize the intercellular spaces of leaf sheaths, culms, and seeds (Rasmussen et al., 2009). Some *Epichloë* species (e.g. *Epichloë festucae* var. *lolii*, *E coenophiala*) are transmitted vertically to the next generation of plant via seeds (Easton, 2007).

Extensive studies have confirmed that *Epichloë* endophyte-grass symbiosis is a defensive mutualism, because *Epichloë* endophytes enhance resistance to biotic stresses (including insects, nematodes, and diseases) (Cook et al., 1991; Prestidge and Gallagher, 2008; Xia et al., 2018) and abiotic stresses (drought, salt, cold and waterlogging) (Chen et al., 2016; Malinowski and Belesky, 2000; Song et al., 2015). *Epichloë* endophytes have been shown in some cases to improve seed germination, plant height, tillers and photosynthetic capacity of host plants (Bao et al., 2019; Chen et al., 2018a; Li et al., 2017; Newman et al., 2003).

In addition, *Epichloë* endophyte infection affects the biomass and morphology of root, although it is distributed in the nutrient-rich above-ground parts of host (Mackie-Dawson, 1999; Wäli et al., 2006). The plant root is an important organ that has multiple functions including acquisition of water and mineral nutrients, perception of environment changes in soil, and assimilation, transformation and synthesis of many substances (such as amino acids, plant hormones, alkaloids, etc.) and interaction with soil microbes (Yin et al., 2014). Growth and development, and metabolic activity of roots directly impact life activities of the plant; root activity includes cellular metabolism and the ability of the plant to interact with soil microbes and absorb nutrients (Brown and Scott, 1984; White et al., 2019). Root exudates are diverse secretions composed of organic acids, amino acids, sugars, phenolic acids, proteins, flavonoids, enzymes, fatty acids, nucleotides, tannins, steroids, terpenoids, alkaloids, polyacetylenes, and vitamins of different classes of primary and secondary compounds (Doan et al. 2017; Inderjit and Weston 2003). C, N, P and other elements affect the synthesis and content of these compounds (Malinowski et al. 1998b; Vengavasi and Pandey 2018). *Epichloë* endophytes are heterotrophic symbiotic fungi absorbing nutrients like amino acids and sugars from host plants (Lemons et al., 2005; Rasmussen et al., 2007). *Epichloë* endophytes have been found to increase the amount and alter the composition of root exudates, which leads to increased microbial activity and nutrient mining from soil around roots (Hosseini et al. 2015; Guo et al. 2015; White et al. 2019). Thus, *Epichloë* endophytes, exclusively in above ground plant parts, affect plant root activity and plant nutrition even though they do not extend into the soil. At present, studies on how endophytes modify nutrient absorption of host plants have been mainly focused on tall fescue (Malinowski et al., 2000; Malinowski et al., 1998a; Rahman and Saiga, 2005) and *Festuca rubra* (Vázquez Aldana et al., 2013, 2014; Zabalgogeazcoa et al., 2006). Meanwhile, other studies have focused on single mineral elements like nitrogen (Wang et al., 2018) and phosphorus (Ren et al., 2007), because of N and P involved in the synthesis of alkaloids (Malinowski et al., 1998b), or some heavy metals (Helander et al., 2011; Monnet et al., 2005). For perennial ryegrass (*Lolium perenne*), there are only two limited reports relating to effects of *Epichloë* endophytes on nutrient absorption, and the objective of the two reports mainly focused on the effects of fertilization and endophyte types on plant nutrient content (Ren et al., 2009; Sotobarajas et al., 2016). However, there is little direct data evidence that *Epichloë* endophytes affect nutrient uptake, accumulation and allocation of leaves and roots under conditions of nutrient deficiency, and the change of positive or negative effects of endophytic fungi on host plant with plants growth over time.

Perennial ryegrass (*L. perenne*) is one of the widely cultivated forage and turf grass species due to its desirable agronomic performance in temperate climates.

L. perenne establishes rapidly and has abundant tillers and high resistance to trampling. *L. perenne* adapts to a wide range of adverse environmental conditions, including saline and alkali soils and soil moisture deficits. Based on these reasons, it is often selected as a model species for stress studies (Sampoux et al., 2013). Both asexual (e.g. *E. festucae* var. *lolii*) and sexual (e.g. *E. typhina*) *Epichloë* endophytes can infect *L. perenne*, but common infections are asexual (Latch et al., 1984). We previously found endophyte infection frequencies of *E. festucae* var. *lolii* in *Lolium perenne* to range from 0% to 100% in the infected plant tillers and seeds of the grass used in the present study (Chen et al., 2018b). After five years of screening (2014–2018 years), we obtained a high-endophyte subpopulation with high endophyte infection rates in tillers and seeds (the average infection rate: 96.5%) (Chen et al., 2020). However, it is not clear whether high endophyte infection enhances performance of the grass in low fertility soils.

We hypothesized that in perennial ryegrass (selection Lanhei No.1) the association with *E. festucae* var. *lolii* increases root metabolic activity, plant nutrient content, plant growth and improves plant survival rate under low nutrient conditions. In this study we set out to evaluate this hypothesis.

Materials and Methods

Plant Materials

Seeds of *L. perenne* selection Lanhei No. 1 were supplied by Lanzhou University. This selection has high disease resistance (Ma et al., 2015; Tian et al., 2008) and cold tolerance (Chen et al., 2020). Seeds with high and low infection frequencies by *E. festucae* var. *lolii* were selected by continuous field planting at the Yuzhong Experimental Station of Lanzhou University (104°12′ E, 35°85′ N, altitude 1400 m), Gansu Province, China, in 2014 to 2018 (Chen et al., 2018b). Seeds were harvested at maturity, and culms and seeds were monitored by staining and microscopic examination to confirm infection rates by the *Epichloë* endophyte (Chen et al., 2018b). High (≥ 95%) and low (≤ 2%) infection rates of individual plants were designated endophyte-infected (E+) or endophyte-free (E-). E+ and E- seeds were threshed and were stored 24 h at 4 °C to break seed dormancy and maintain endophyte viability.

Experimental Design

A pot experiment was carried out from 10th March to 20th September 2018 in the greenhouse. E+ and E- seeds were planted (broadcast sowing) in trays (50 cm length × 30 cm width), one half of each tray for E+ and the other half for E-, totaling 50 trays, and these trays filled with vermiculite (1.0 kg) that had been previously sterilized at 150 °C for 24 h. After 15 days, 40 trays that contained E+ and E- seedlings were selected. Trays were irrigated with water as needed. The trays had a randomized position in within a variable temperature greenhouse (16 h light/8 h dark, 25 °C day/ 20 °C night, a light intensity of 800 μmol m^{-2} s^{-1} and 60% relative humidity).

E+ and E- seedlings were grown for different periods of time (0 d, 45 d, 90 d, 135 d, 180 d) in sterilized vermiculite, and there were 7 replicates for each time gradient, respectively. During the experiment, seedlings were not fertilized or supplied nutrient solution. Every third day, trays were irrigated with equal volumes of water (1.5 L) to maintain consistent stress conditions, and the position of each tray was changed arbitrarily.

Plant Survival Rate Calculation (Proportion of the shoot That Remained Alive)

After 0 d, 45 d, 90 d, 135 d and 180 d of plant growth, the length and width of every living plant of E+ and E- in every tray was measured before harvest by using a ruler, and the area of each living plant of E+ and E- was calculated according to the length and width of E+ and E-, respectively. The plant survival area of E+ and E- in every tray was calculated by calculating the area of each living plant of E+ and E-. The plant survival rate (PSR) was calculated using the following equation based on the measured total area of plants: PSR (%) = (PSA/HTA) × 100.

Where PSA is the plant survival area (m^2), and HTA is the total area (m^2).

Root Metabolic Activity and Plant Biomass Measurements

All the leaves above vermiculite surface were harvested on 0, 45, 90, 135 and 180 days after the start of seedling emergence, respectively, and all roots were washed with distilled water to remove vermiculite. Root activity was determined by the method of triphenyl tetrazolium chloride (TTC) (Lee et al., 2006). Briefly, 0.5 g root sample was taken into 10 mL beaker and 10 mL 0.4% TTC solution was added along with 10 mL phosphoric acid buffer (pH = 7.0). Roots were sufficiently immersed in the mixed solution and kept in the dark at 37 °C for 3 h, then added 2 mL 1 mol/L sulfuric acid (H_2SO_4) to stop reaction. The water of the root that was taken out from the mixed solution was absorbed, and then ground with 4 mL ethyl acetate and a small amount of quartz sand in a mortar to get triphenyl azan (TF). The absorbance of TF and blank experiment was measured at 485 nm, and the root TTC reductive amount was calculated by using the standard curve. Root metabolic activity was calculated using the following formula: TTC intensities ($mg.g^{-1}.h^{-1}$) = TTC reductive amounts (mg)/[root weight (g) × time (h)]. To determine plant dry weight, all plants samples were oven-dried at 80 °C until a constant weight.

Chemical Analysis

For chemical analyses, all dried tissues were weighed using a balance, and ground in a MM400 ball mill (Retsch, Germany) to obtain homogenous samples.

Plant organic carbon (OC) content was measured using K_2CrO_7-H_2SO_4 oxidation method (oil bath at 180 °C for 5 min, followed by titration with $FeSO_4$) (Tanveer et al., 2014). Leaves and roots samples were determined for total carbon (TC) by using a CHNS/O analyzer (Flash EA 1112 Serues, Italy). To determine the total nitrogen (N) and total phosphorus (P) contents in leaves and roots tissues, each sample was digested with H_2SO_4 under catalyzed condition ($CuSO_4$:K_2SO_4: 1:10 mixture) on a digestion block at 420 °C for 1 h, and then the concentrations of N and P were analyzed by flow injection system (FIAstar 5000 Analyzer, Foss, Denmark). Then, the ratios of total C, N and P in leaves and roots (C:N, C:P and N:P) were calculated.

For mineral content, dried samples of leaves and roots tissues were ashed (450 °C), and ashes were dissolved in HNO_3: H_2SO_4: $HClO_4$ (8:1:1). The concentrations of Ca, Fe, Mn, Zn, Cu and Mg were determined by flame atomic absorption spectrometer (Thermo ICE 3300, Germany), and Na and K were determined using a flame spectrophotometer (Sherwood M410, Britain).

Statistical Analysis

Effect of endophyte status (E: E+ and E-) and plant growth time (T: 0d, 45d, 90d, 135d and 180d) on plant survival rate, root metabolic activity, plant biomass and nutrient content parameters were analyzed with a two-way ANOVA. When a significant effect was detected, the differences between means of lines were assessed using Tukey's-b(k) test at $P = 0.05$. All analyses of variance (ANOVA) were performed using SPSS statistical software (Version 20.0, Inc., Chicago, IL).

To assess the contributions of endophyte infection to responses of plant growth (biomass and root metabolic activity) and plant nutrition (macro-elements and trace elements) in leaves and roots, and to analyze and explain the ways that the effects of endophyte affects plant survival rate, we used structural equation modeling (SEM). The primary advantage of SEM is to model multivariate relations and to evaluate multivariate hypotheses between variables by transforming hypothetical causal relationships into a pattern of expected statistical relationships in the data (Grace et al., 2010). Prior to the SEM procedure, we filtered the response variables for biomass, plant nutrition through correlation analysis. We used the goodness of fit chi-square statistic and its associated *P* value to assess the model to the data. The fit of the model was considered to be goodness when $0 \le \chi^2/df \le 2$ and $0.05 < P \le 1$ (Grace et al., 2010). If a model was not established, connections were iteratively added until model acceptance was qualified and additional links understood as new discoveries of processes previously unanticipated (Grace, 2006). A large P value (> 0.05) associated with the chi-square value indicates that the covariance structure of the data does not differ significantly from the expected (Grace, 2006). SEM analyses were performed using AMOS 21 (Amos Development Corporation, Chicago, IL, USA) (Grace, 2006).

Result

Plant Survival Rate and Root Metabolic Activity

The survival rate of *L. perenne* was significantly affected by *Epichloë* endophyte infection, growth time and their interaction (Table 1). Plant growth time negatively affected the plant survival rate of plants (Fig. 1). The survival rate of E+ plants was significantly higher than that of E- plants when growth time was 45 d, 90 d and 135 d (Fig. 1). The survival rate of E- plants at the 0–45 d was significantly lower than in E+ plants (31.4%) (Fig. 1). However, withered and yellowed leaves in E+ plants significantly increased at period 45 d to 90 d, but continued to have higher the survival rates than E- plants (Fig. 1).

Root metabolic activity of the host was significantly affected by *Epichloë* endophyte infection and plant growth time, but the interaction between *Epichloë* endophyte infection and plant growth time was not significant (Table 1). Comparisons of E+ and E- plants demonstrated that presence of the endophyte significantly positively influenced root metabolic activity (Table 1, Fig. 2A). Root metabolic activity of *L. perenne* plants declined with increased plant growth time, and root metabolic activity was significantly different among different growth times (Table 1, Fig. 2B).

Dry weight in Leaves and Roots

Dry weights of leaves and roots were significantly affected by endophyte infection and plant growth time, but their interaction did not have a significant effect on dry weight in leaves ($P = 0.662$) (Table 1). The dry weight of leaves was higher in E+ than in E- plants (Fig. 3A). Comparing the plants under different growth times, dry weights of leaves at 0 to 90d were significantly lower than that of plants at 135d and 180d, although the plants were mainly composed of dead tissue at these time-periods (135d and 180d); plant survival rate of E+ and E- was 26.1% and 16.8% at 135d, and was 1.8% and 1.3% at 180d (Fig. 3B). The presence of the *Epichloë* endophyte significantly increased the dry weight of roots at growth periods 45d, 90 d, 135d and 180d, although plants were mainly composed of dead tissue at 135d and 180d (Fig. 3C). A maximum peak emerged for dry weight in roots of E+ and E- plants at 135 days (Fig. 3C).

Nutrient content of plants

C, N and P contents

The concentrations of OC, TC and N in leaves and roots were significantly affected by *Epichloë* endophyte infection, plant growth time and their interaction (Table 2). In general, the OC, TC and N contents significantly reduced with plant growth time (Fig. 4). *Epichloë*-infected (E+) plants had significantly higher content of OC in leaves and roots than E- plants at the 45 d and 90 d, but between E+ and E- plants at 135d and 180d did not have a significant difference on OC content in leaves and roots (Fig. 4A and B). The TC content in leaves was higher in E+ than in E- plants at 45d and 90d (Fig. 4C). For roots, the TC content of E+ plants was significantly higher than of E- plants at 45d. (Fig. 4D). The E+ plants had a significantly higher N content than E- plants in leaves and roots at 45d and 90d of plant growth (Fig. 4E and F). In addition, N content was higher in leaves than in roots (Fig. 4E and F). The OC, TC and N contents of leaves and roots were lower in E+ than in E- plants at 135d and 180d although there was no significant difference between E+ and E- plants (Fig. 4A, B, C, D, E and F).

The P content of leaves was higher in E+ than in E- plants at 45d and 90d, and it was higher in E- than in E+ plants at 135d and 180d although in this case differences were not significant (Fig. 5A). The interaction between *Epichloë* endophyte infection and plant growth time was not significantly affected in P content of roots ($P = 0.887$) (Table 2). The P content of roots was higher in E+ than in E- plants (Fig. 5B). Comparing the plants under different growth times, the P content in roots of plants at 0d and 45d were significantly higher than that of plants at 90d, 135d and 180d (Fig. 5C).

Stoichiometric ratios of total C, N and P

Epichloë endophyte infection did not have significant effects on the C:N ratio of leaves ($P = 0.106$), but the interaction between *Epichloë* endophyte and growth time was significantly affected in C:N ratio (Table 3). When plant growth time was 45 d and 90 d, lower C:N ratio was found in E+ than in E- plants in leaves and roots (Fig. 6A and B), but E+ plants had significantly higher C:N ratio in leaves than E- plants at the 135d (Fig. 6A). The C:P ratio at different growth times was higher in E- than in E+ plants both in leaves and roots, but there was no difference between E+ and E- plants in the control (0 d) (Fig. 6C and D). In the leaves, the differences in the N:P ratio was significantly different between E+ and E- plants at 45d to 135d (Fig. 6E). The N:P ratio in

Table 1 Two-way ANOVA for the effects of endophyte status (E) and growth time (T) on plant survival rate, root metabolic activity and dry weight of leaves and roots of *Lolium perenne*

Source	df	Plant survival rate		Root metabolic activity		Dry weight			
						leaves		roots	
		F	*P*	F	*P*	F	*P*	F	*P*
E	1	73.0	<0.001	6.4	0.007	6.9	0.016	208.6	<0.001
T	4	73.0	<0.001	37.9	<0.001	9.9	<0.001	1196.9	<0.001
E x T	1	8.5	0.006	0.9	0.452	0.6	0.662	24.8	0.009

F is F-value, statistical value of F-test;

The same below of table.

roots presented a similar regular pattern to the C:P ratio as plants grew (Fig. 6F).

Na, K, Ca and Mg Contents

The Na content of leaves and roots of *L. perenne* was not significantly affected by *Epichloë* endophyte infection ($P = 0.694$ and $P = 0.879$) and neither the interaction between *Epichloë* endophyte infection and plant growth time ($P = 0.341$ and $P = 0.436$) (Table 4, Fig. 7A and B). The presence of the endophyte resulted in a significant difference in the K content of ryegrass (Table 4), where E+ plants had more K in leaves by approximately 30% than E- plants at the 45 d (Fig. 7C). The K content in roots was higher in E+ than in E- plants at 45d and 90d of plant growth, and E+ plants contained approximately 34% and 25% more K content in roots than E- plants at the 45d and 90d, respectively (Fig. 7D).

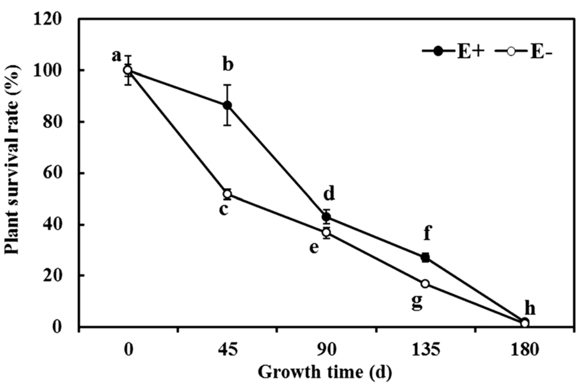

Fig. 1 Plant survival rate of *Lolium perenne* with (E+) and without (E-) *Epichloë* at different growth times. Values are means±standard error. For each graph, different lowercase letters indicate significant difference ($P < 0.05$) between means. The same below of figures

However, the K content of leaves at 135d and 180d of plant growth, and of roots at 180d of plant growth did not differ between E+ and E- plants (Fig. 7C and D). E+ plants had significantly higher Ca content of leaves than E- plants at 45 d, 90 d and 135 d of growth (Fig. 7E). In roots, the Ca content of plants was not significantly affected by *Epichloë* endophyte infection or their interaction (Table 4, Fig. 7F). Endophyte infection increased the absorption of K content into leaves and roots, and increased the absorption and accumulation of Ca content in leavers under conditions of nutrient deficiency.

The presence of the endophyte significantly increased Mg content of leaves ($P = 0.021$) (Table 4, Fig. 8A), but was not significantly affected by the interaction between *Epichloë* endophyte infection and plant growth time ($P = 0.635$) (Table 4). The Mg content of leaves significantly increased with plant growth time (Fig. 8B). The Mg content of roots was significantly greater in E+ plants than in E- plants at 45 d and 90 d, and a maximum peak occurred at 90 days (Fig. 8C). Differences between E+ and E- plants in Mg content of roots at 135d and 180d were not significant (Fig. 8C).

Fe, Mn, Zn and Cu Contents

E+ plants had significantly higher Fe content in leaves than E- plants at 45d and 90d (Fig. 9A). For roots, Fe content was significantly higher in E+ than E- plants at 45 d, 90 d and 135 d, and a maximum peak occurred at 90 d (Fig. 9B). The Mn content of leaves and root was significantly affected by the interaction between *Epichloë* and growth time (Table 5, Fig. 9C and D). The Mn content of E+ plants was significantly higher than of E- in leaves at 45d to 135d of plant growth (Fig. 9C), and in roots at 45d and a maximum peak in Mn

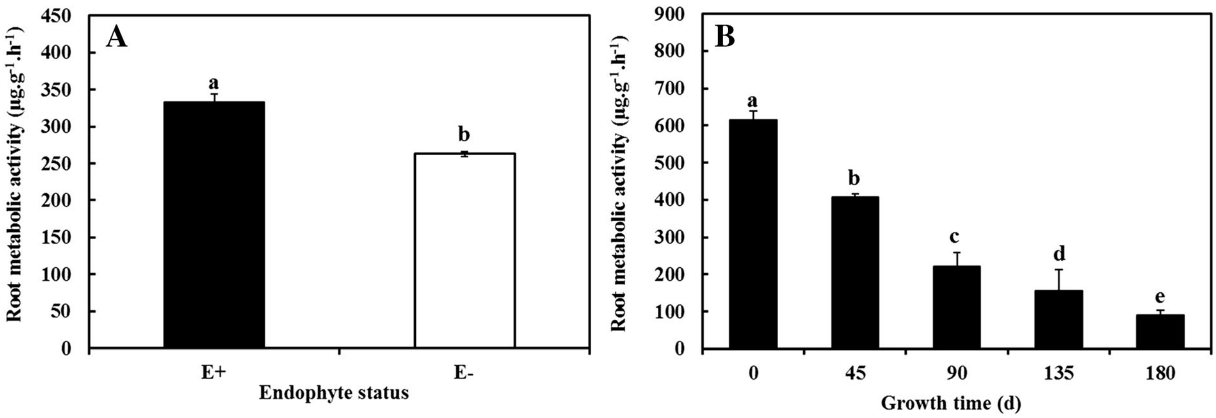

Fig. 2 Root metabolic activity in *Epichloë*-infected (E+) and *Epichloë*-free (E-) plants of *Lolium perenne* (A), and at different growth times (B)

content occurred at 90 d in roots (Fig. 9D). At plant growth times of 45 d, 90 d and 135 d, E+ plants had significantly higher Zn content in leaves than E- plants (Fig. 9E). However, the Zn content of *L. perenne* in roots was not significantly affected by endophyte infection neither the interaction between endophyte and growth time (Table 5, Fig. 9F). The Fe content of leaves at 135d and 180d, and in roots at 180d of plant growth, and Mn content of roots at 180d was lower in E+ than in E- plants (Fig. 9A, B and D).

E+ plants had significantly higher Cu content in leaves at 45d and 90d of plant growth, but differences between E+ and E- plants in Cu content of leaves at 135d and 180d were not significant (Fig. 10A). The presence of *Epichloë* endophyte significantly reduced the Cu content in roots ($P < 0.001$), but there was not a significant endophyte × growth time interaction ($P = 0.976$) (Table 5, Fig. 10B). Comparing the plants under different growth times, the Cu content in roots of plants at 180d was significantly higher than that of plants at 0d, 45d, 90d and 135d (Fig. 10C). The presence of endophyte increased the absorption of Cu content into leaves under conditions of nutrient deficiency, but overall reduced the accumulation of Cu in roots.

SEM Leaves-Model for the Effect of Endophyte on Plant Survival Rate

The overall SEM successfully explained 97.8% of the variation in plant survival rate (Fig. 11). Endophyte infection had a positive association with C, N, P, K, Ca and the micronutrient Mn, and dry weight of leaves, and had a negative association with Mg and Cu content of leaves (Fig. 12). Endophyte infection had a significant positive direct, or indirect effect on plant survival rate through a positive impact on C, N, P, K, Ca, Mn content and dry weight of leaves, and negative impact on Mg and Cu (Fig. 11). Plant survival rate was positively related to C, N, P, K, Ca, Mn, and Mg content of leaves, and was negatively related to Cu content and dry weight of leaves (Fig. 11).

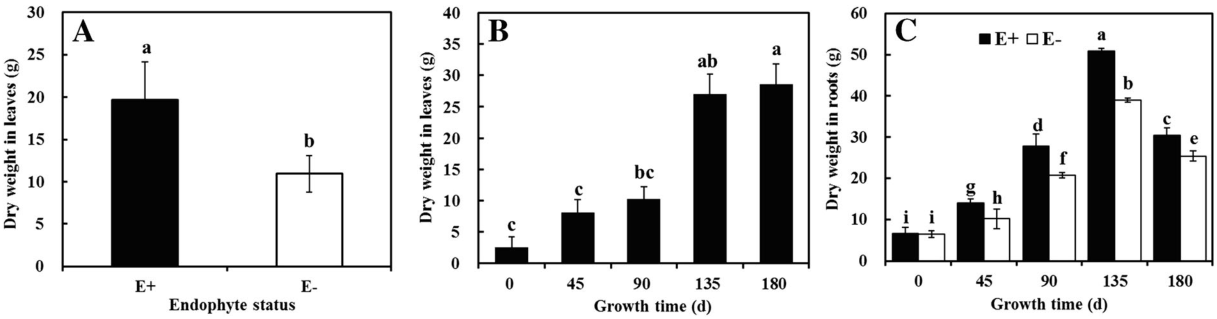

Fig. 3 Leaves dry weight of *Lolium perenne* in *Epichloë*-infected (E+) and *Epichloë*-free (E-) plants (A), and at different growth times (B), and dry weight of E+ and E- plants in roots at different growth times (C)

Table 2 Two-way ANOVA for the effects of endophyte status (E) and growth time (T) on OC, TC, N and P contents of leaves and roots of *Lolium perenne*

Source	df	OC content				TC content				N content				P content			
		leaves		roots		leaves		roots		leaves		roots		leaves		roots	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
E	1	4.8	0.041	4.6	0.045	11.7	0.003	4.5	0.046	4.5	0.031	9.0	0.007	17.6	0.002	7.5	0.008
T	4	107.6	<0.001	95.8	<0.001	161.9	<0.001	162.0	<0.001	357.7	<0.001	550.8	<0.001	268.0	<0.001	180.3	<0.001
E x T	1	4.9	<0.001	3.3	0.03	9.1	0.008	4.3	0.006	3.1	0.034	9.1	0.007	4.3	0.005	0.3	0.887

K and Mn content of leaves was a key factor affecting plant survival rate (Fig. 11). Endophyte infection was shown to influence positively the K content of leaves through a positive direct effect on P content of leaves [standardized path coefficients (SPC) of 0.305, $P < 0.01$], then K of leaves produced positive direct effects on plant survival rate (SPC of 0.663, $P < 0.001$), and indirect positive effects on plant survival rate via affecting dry weight (SPC of −0.326 and − 0.548, respectively, $P < 0.001$ and $P < 0.01$), Cu (SPC of −0.520 and − 0.161, respectively, $P < 0.001$ and $P < 0.01$), Mg (SPC of −0.826 and 1.010, both $P < 0.001$; SPC of −0.826, 0.695, 0.559 and − 0.548, $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.01$) and Ca (SPC of −0.690, −0.329 and 0.281, $P < 0.001$, $P < 0.001$ and $P < 0.05$; SPC of −0.690, −0.329, −0.710 and − .0161, $P < 0.001$, $P < 0.001$, $P < 0.001$and $P < 0.01$) (Fig. 11).

Endophyte infection had positive and indirect effects on Mn content through positive effects on OC (SPC of 0.435, $P < 0.001$) and P (SPC of 0.209, $P < 0.05$), and K (SPC of −0.514, $P < 0.001$) and Ca (SPC of −0.329, $P < 0.001$) content showed direct negative effects on Mn content, but Mg (SPC of 0.695, $P < 0.001$) content had direct positive affect on Mn content (Fig. 11). The Mn content of leaves also showed direct positive effects on plant survival rate (SPC of 0.281, $P < 0.05$), and indirect effects on plant survival rate by affecting Cu content (SPC of −0.710 and − 0.161, respectively, $P < 0.001$ and $P < 0.01$), and dry weight of leaves (SPC of 0.559 and − 0.548, respectively, $P < 0.001$ and $P < 0.01$) (Fig.11).

SEM Roots-Model for the Effect of Endophyte on Plant Survival Rate

The SEM was successful in explaining the variance in plant survival rate ($R^2 = 98\%$), while plant survival rate was not positively directly related to endophyte infection by roots SEM (Fig. 13). Endophyte infection had a positive association with C, N, P, K, root metabolic activity and dry weight of leaves and roots, and had a negative association with Ca and Cu content of roots (Fig. 14). Endophyte infection only had a significant positive indirect effect on plant survival rate via positive impact on C, N, P and K in roots, root metabolic activity and dry weight of leaves and roots, and negative impact on the Ca and Cu content of roots, but did not positively directly affect plant survival rate (Fig. 13).

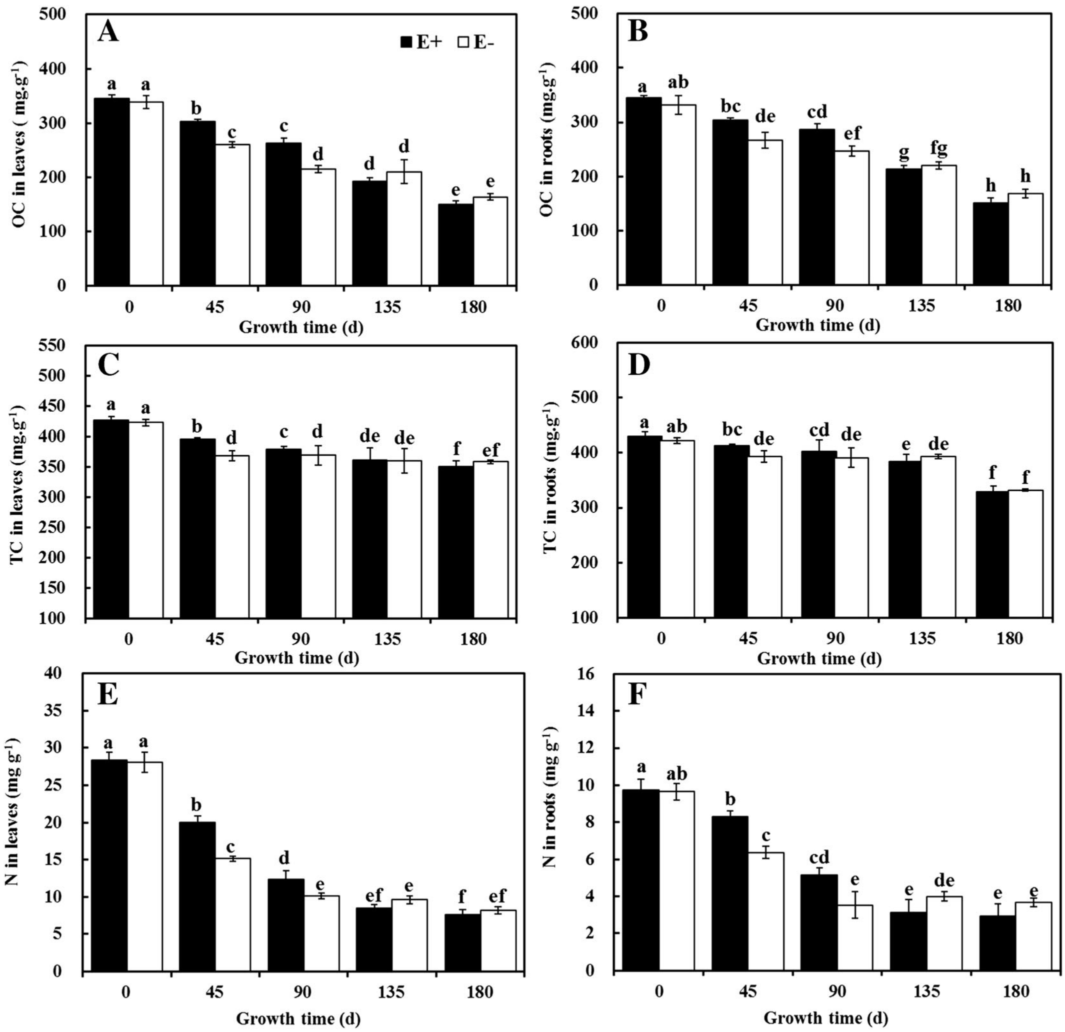

Fig. 4 OC, TC and N contents in leaves and roots of *Lolium perenne* plants with (E+) and without (E-) the *Epichloë* endophyte at different growth times (A, B, C, D, E and F)

In the SEM, root activity, K in roots, and roots dry weight were main factors affecting increased plant survival due to endophyte infection (Fig. 13). Endophyte infection had positive effects on plant survival

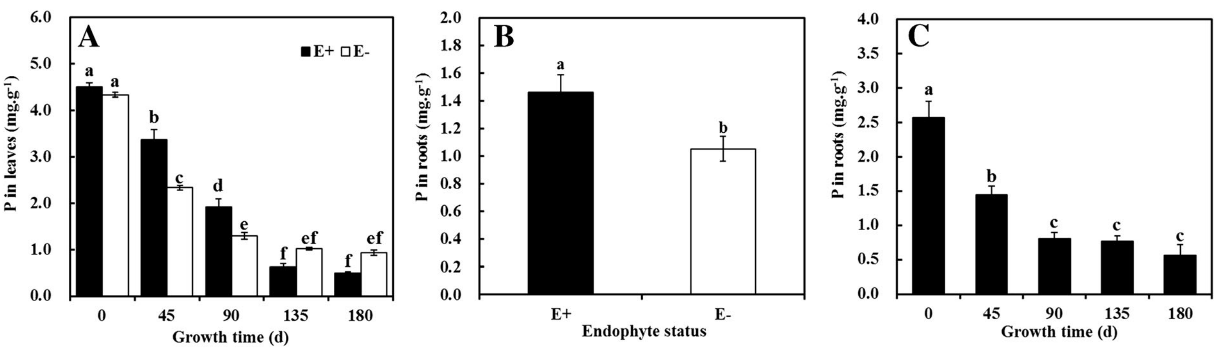

Fig. 5 P content of leaves of *Lolium perenne* plants with (E+) and without (E-) the *Epichloë* endophyte at different growth times (A), and the P content of roots (B), and at different growth times (C)

Table 3 Two-way ANOVA for the effects of endophyte status (E) and growth time (T) on C:N, C:P and N:P ratios in leaves and roots of *Lolium perenne*

Source	df	C:N				C:P				N:P			
		leaves		roots		leaves		roots		leaves		roots	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
E	1	0.9	0.106	11.4	0.003	265.3	<0.001	331.1	<0.001	46.2	<0.001	41.9	<0.001
T	4	104.4	<0.001	114.4	<0.001	1941.1	<0.001	927.4	<0.001	26.3	<0.001	35.8	<0.001
E x T	1	6.2	0.002	5.9	0.003	21.1	<0.001	62.3	<0.001	5.4	0.004	3.6	0.022

rate via direct positive effects on root metabolic activity (yellow arrow, SPC of 0.127, $P < 0.05$), K in roots (red arrow, SPC of 0.172, $P < 0.001$), and root dry weight (green arrow, SPC of 0.465, $P < 0.001$), then three variables produced direct positive effects on plant survival rate; see yellow path (SPC of 0.580, $P < 0.001$), red path (SPC of 0.382, $P < 0.001$), and green path (SPC of 0.163, $P < 0.01$), respectively (Fig. 13). Another path of roots, K (purple arrow, SPC of −0.670, 0.410 and − 0.191, $P < 0.001$, $P < 0.001$ and $P < 0.01$), and root metabolic activity (light blue arrow, SPC of −0.714 and − 0.191, $P < 0.001$ and $P < 0.01$), also showed a significant indirect effect on plant survival rate (Fig. 13).

Discussion

In this study, we found that *E. festucae* var. *lolii* significantly influenced the fresh and dry weights, root metabolic activity, nutrient content, and plant survival rate of *L. perenne*, and the effects of plant growth time on these parameters was higher than of *Epichloë* endophyte infection. Our results indicate that *Epichloë* endophyte infection can alleviate nutrient deficiency stress in the host plant by promoting root metabolic activity, and decreasing the stoichiometric ratios of C:N, C:P and N:P in leaves and roots, and Cu content of roots, or increasing the concentration of OC, TC, N, P, K, Ca, Mg, Fe, Mn and dry weight of leaves and roots, and Cu content of leaves and root activity in the absence of fertilization. The effect of *Epichloë* endophyte infection on nutrient content was dependent on the host growth time. The Mn content of leaves, K content in leaves and roots, the metabolic activity and dry weight of roots play an important role in enhancing plant survival rate.

Effects of *Epichloë* Endophyte Infection on Root Metabolic Activity and Biomass of Leaves and Roots

Root metabolic activity refers to the strength of root metabolic activity (including growth, and nutrient exudation for rhizobacterial symbiosis and nutrient absorption) (Xu and Huang, 2006). Several studies have suggested that endophyte increases root growth [e.g., root length (Malinowski et al., 1998b), root-shoot ratio (Wang et al., 2017b), some enzyme activities in roots (Wang et al., 2018), root nutrient exudation and altered chemical constituents of roots (Malinowski et al., 2004)]. Further, increased root activity may reflect stimulation of root symbiosis with rhizobacteria that function in roots and the soil in nutrient mining to increase absorption of soil nutrients by roots (White et al., 2018, 2019). Our results showed that the presence of *E. festucae* var. *lolii* increased root metabolic activity in the most nutrient demanding early periods of plant growth, but the positive effects of *Epichloë* endophyte on root metabolic activity was attenuated with plant growth time. Nutrient content is important for growth and development of the plant root system, and significantly affects root metabolic activity (Mackie-Dawson, 1999; Ruiz Herrera et al., 2015). N has been identified as one of the most important indexes for improving absorption efficiency of root systems (Chapman et al., 2012; Garnett et al., 2009). A previous study also demonstrated that improving availability and supply of P and K is helpful in promotion of the physiological metabolism of crop roots (Williamson et al., 2001; Zhang et al., 2009). Malinowski et al. (2000) reported that leaf-associated endophytes could affect root metabolic activity in tall fescue by increasing mineral uptake and transport to shoots. We have also shown increases in root metabolic activity in *Epichloë* infected *L. perenne* plants. The endophyte had positively direct

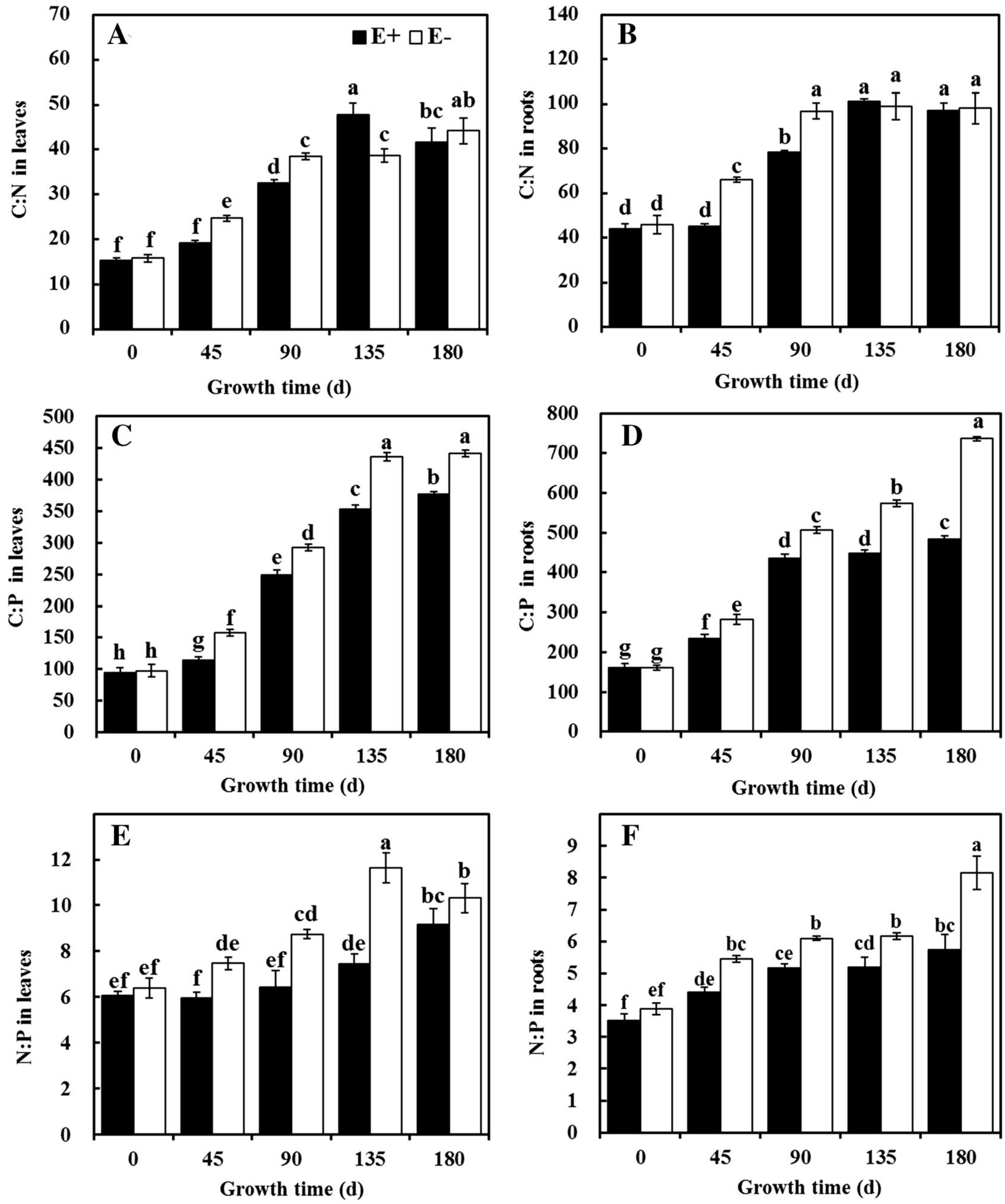

Fig. 6 Stoichiometric ratios of total C, N and P in *Lolium perenne* plants with (E+) and without (E-) *Epichloë* at different growth times at leaves and roots

effects on root metabolic activity in *L. perenne* and had an indirect effect by altering K content in roots, and as such benefits the survival of host plants under conditions of nutrient deficiency.

A field study showed E+ plants have significantly higher dry matter yield than E- plants under a low water conditions (West et al., 1988). In *L. perenne*, the dry weights of leaves and roots were significantly affected by *E. festucae* var. *lolii* infection status (E+, E-) under low fertility. Our results agree with those of other studies showing significant *Epichloë* endophyte effects on plant growth (Oberhofer et al., 2014; Xia et al., 2016). Foliar endophytes of grasses have been shown to affect below-ground processes upon their host death by altering quality of the litter, or the decomposer community and the microenvironment for decomposition (Omacini

Table 4 Two-way ANOVA for the effects of endophyte status (E) and growth time (T) on Na, K, Ca and Mg contents of leaves and roots of *Lolium perenne*

Source	df	Na content				K content				Ca content				Mg content			
		leaves		roots		leaves		roots		leaves		roots		leaves		roots	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
E	1	0.5	0.694	1.8	0.879	11.7	0.008	6.0	0.008	36.5	<0.001	2.2	0.468	6.6	0.021	12.3	0.007
T	4	186.2	<0.001	26.4	<0.001	161.9	<0.001	1597.8	<0.001	116.7	<0.001	66.4	<0.001	34.7	<0.001	212.2	<0.001
E x T	1	1.9	0.341	1.5	0.436	9.1	0.003	17.9	0.006	3.6	0.034	1.3	0.678	2.4	0.635	13.0	0.008

et al., 2004; Purahong and Hyde, 2011). Our results showed that *Epichloë* endophyte infected plants have higher root metabolic activity, and higher dry weight of leaves and roots than endophyte-free plants under low fertility conditions. Other studies reported that nutrients are critical for increased plant yield (Fageria et al., 2008). From the SEM (Figs. 11 and 13), the presence of *E. festucae* var. *lolii* affected the dry weight in leaves and roots by affecting nutrient contents (including, OC, N, P, K, Ca, Mg and Mn in leaves, or OC, N, K and Ca in roots). In addition, roots are important organs of interaction with symbiotic microbes that function in soil nutrient mobilization, increasing availability of nutrients for absorption by roots, and root growth and development directly affect the growth of above-ground tissues and crop yield (Unger, 1979; Wu and Qiu, 1992). In *L. perenne*, the dry weight in leaves and roots has negative association with root activity (Fig. 13), it is possible that plant survival decreased with plant growth, even was very low (less than 20%) after 135d and 180 days led to reduced root activity under the very-low-fertility conditions, but it makes the plants obtain accumulation of the dry weight in leaves and roots by absorbing nutrients from water and vermiculite in the early growth stage.

Effects of *Epichloë* Endophyte Infection on Nutrient Content of Plants

When soil fertility is poor, plant tissues correspondingly have lower content of most nutrients, including N, P, K, Mg, Cu and Zn (Marschner, 1995). Vázquez Aldana et al. (2013) reported that E+ plants of *L. perenne* showed higher P, N and Zn in shoots, and higher Ca, Zn and Mg in roots, compared to E- plants. In addition, many studies have demonstrated that *Epichloë* infection can increase host nutrient uptake of nutrients like Mo and Zn (Malinowski et al., 2004), Zn and Ca in tall fescue (Malinowski et al., 2000), P, Mg, Mn and Fe in *F. rubra* (Zabalgogeazcoa et al., 2006), and P content in *Achnatherum sibiricum* (Li et al., 2012). Infected *L. perenne* plants in early growth stages (45d and 90d), compared to uninfected plants in this experiment, suggests a positive role of *E. festucae* var. *lolii* in the plant's response to growth under low fertility conditions. These elements (such as C, N, P and K) are involved in material synthesis, energy metabolism and material transport, etc. It is possible that higher the contents of C, N, P, K, Ca, Mg, Mn, Fe and Cu in

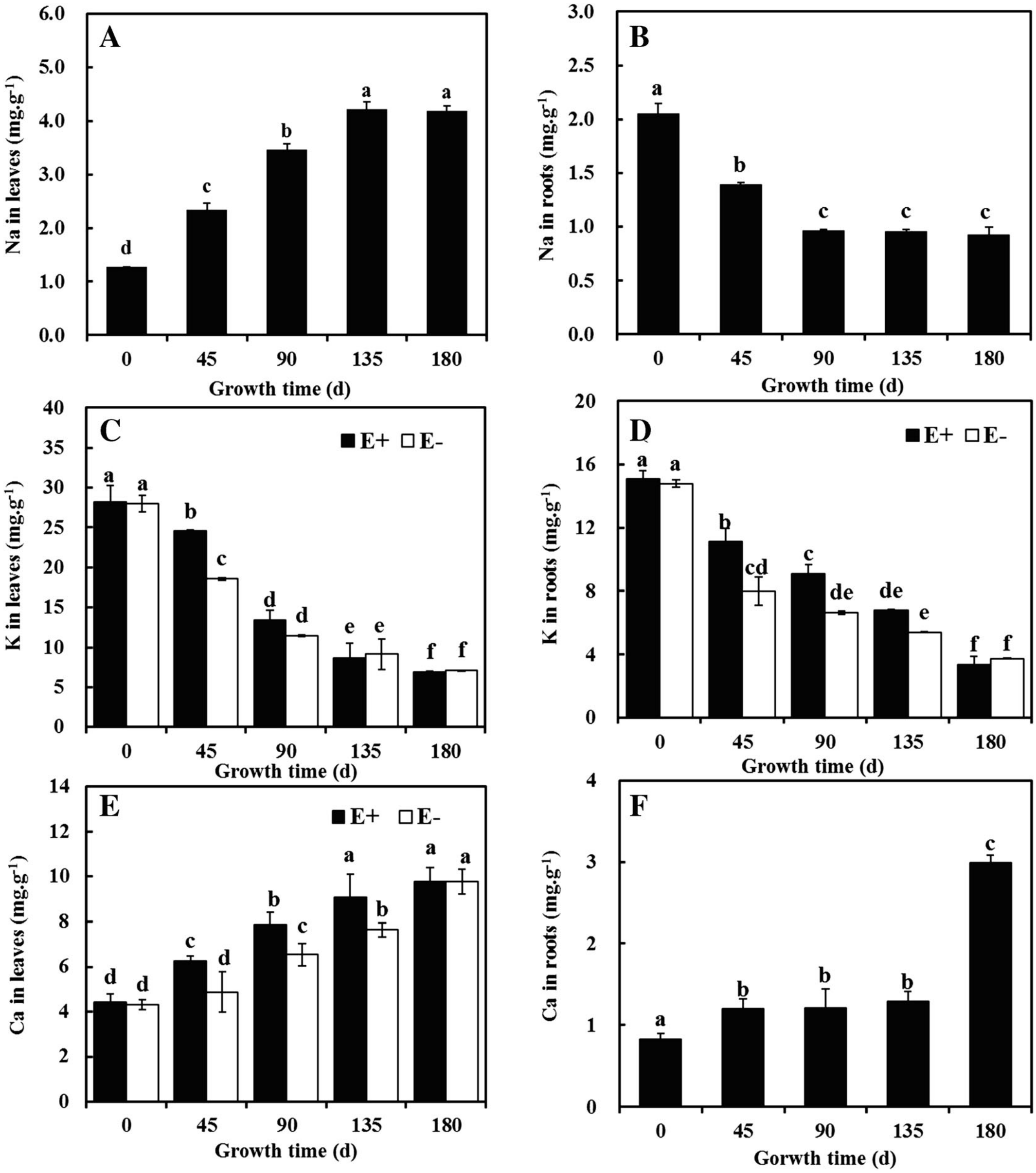

Fig. 7 Na content of leaves and roots and Ca content of roots of *Lolium perenne* at different growth times (A, B and F), and the K content of leaves and roots and Ca content of leaves of plants with (E+) and without (E-) *Epichloë* at different growth times (C, D and E)

leaves, and of C, N, P, K, Mg and Fe in roots at early growth stages is because of *Epichloë* infection affected metabolic processes of synthesis and metabolism. Some studies have reported that *Epichloë* infection had no significant effects on the content of nutrient elements (P, K, Ca, Mg, Na, Mn, Fe, Zn, Cu) (Ren et al., 2009). We found no effect of *E. festucae* var. *lolii* on C, N, P, Na, K, Mg and Zn in later stages of host growth (135d to 180d). This may be the result of decreased demand for nutrients in older plants where growth rates decrease; and this may have permitted E- plants to 'catch-up' to E+ plants in nutrient content. Another possibility is that nutrients in the medium of E+ plants were exhausted. In the present study we found that *Epichloë* infected *L. perenne* decreased the concentrations of Cu in roots compared to uninfected plants, which is consistent with other studies reporting that *Epichloë* negatively affects some nutrient elements (Dennis et al., 1998; Ren et al.,

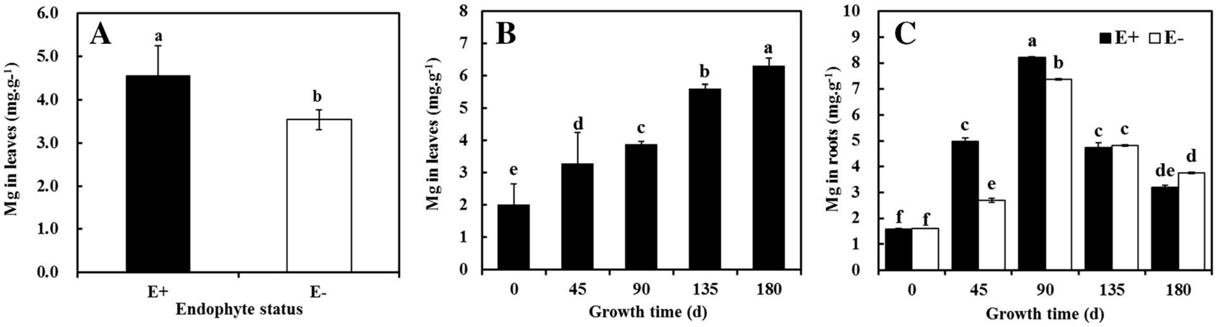

Fig. 8 Mg content of leaves of *Lolium perenne* in *Epichloë*-infected (E+) and *Epichloë*-free (E-) plants (A), and at different growth times (B), and Mg content of roots of plants with (E+) and without (E-) *Epichloë* at different growth times (C)

2007). In addition, the results of our study showed that percent of macronutrients N, P and K in leaves decreased with growth time, meanwhile Ca, Mg, Na and micronutrients increased. This could be due to the fact that N, P and K are used heavily in early rapid plant growth to construct plant structure, while other nutrients may be more heavily needed as physiological activities commence in more mature plant tissues.

C, N and P are essential elements, which are the foundation of biochemical compositions of all life on earth (Guan and Wen, 2011; Vrede et al., 2004). Some studies have demonstrated that the presence of endophytes can affect the C, N and P contents of host plants (Chen et al., 2018a). Lyons et al. (1990) reported endophyte infection affects the accumulation of organic and inorganic phosphorus in leaves and sheaths of tall fescue. In nitrogen deficiency, E+ plants had higher nitrogen fertilizer utilization efficiency than E- plants (Lewis., 2015). Malinowski et al. (1998b) observed higher concentrations of P in above-ground and below-ground structures in E+ plants compared to E- plants in low phosphorus conditions. From the SEM (Fig. 11), *Epichloë* infection had significant effects on the concentrations of OC, N and P of leaves. *Epichloë* endophytes are not involved in photosynthesis, but they consume fixed carbon (Kuldau and Bacon., 2008). N and P are involved in alkaloid synthesis by the fungal endophyte (Malinowski et al., 1998b). It has been previously shown that *Epichloë* infection improves photosynthesis of host plants (Rozpądek et al., 2015). In roots, *E. festucae* var. *lolii* had significant, direct effects on only OC content, but had no direct effects on N and P content (Fig. 13), and the *Epichloë* endophytes had only indirect effects on plant survival by affecting OC and N contents, most likely because *Epichloë* endophytes serve as a nutrient sink in that they are heterotrophic symbiotic fungi absorbing carbohydrates (e.g sugars) from host plants (Rasmussen et al., 2010), which are stored in the basal parts of leaf sheaths and in roots (Rahman and Saiga, 2005), and endophytes are only present in aerial parts (Müller, 2003).

It has been reported that there is an intrinsic connection between tissue elemental stoichiometry and growth rate of plants (Reef et al., 2010). The growth rate hypothesis (GRH) holds that organisms can adapt to change of growth rate by changing their C:N:P ratios in the process of growth and development, and higher growth rates are related to lower C:N, C:P and N:P ratios (Chen et al., 2010; Sardans et al., 2012). Lower C:N and C:P ratios in plant leaves indicate that organisms have a stronger ability to absorb nutrients and assimilate C (Vitousek, 1982; Wardle et al., 2004). Our data shows that the presence of *Epichloë* endophyte in ryegrass plants decreased the C:N and C:P ratios in leaves and roots which could indirectly explain why *Epichloë* infected plants had higher biomass under low fertility conditions, as compared to E- plants. The value of N:P ratio in plants can be used as an important index to judge the adaptation of plant growth to nutrient supply (Wassen et al., 2010). Our results indicated that the growth of E+ and E- plants under low fertility conditions was more restricted by N (N:$P < 14$), which is necessary for plants to have a strong photosynthetic capacity under higher growth rates (Virgona and Farquhar, 1996). The N:P ratio of E+ plants was significantly lower than E- plants, which is consistent with some studies demonstrating that the *Epichloë* endophyte significantly affects plant growth by decreasing the N:P ratio (Chen et al., 2018a).

K is an essential nutrient for plant growth and metabolism, and its availability is essential in enzyme activation, osmoregulation (water utilization),

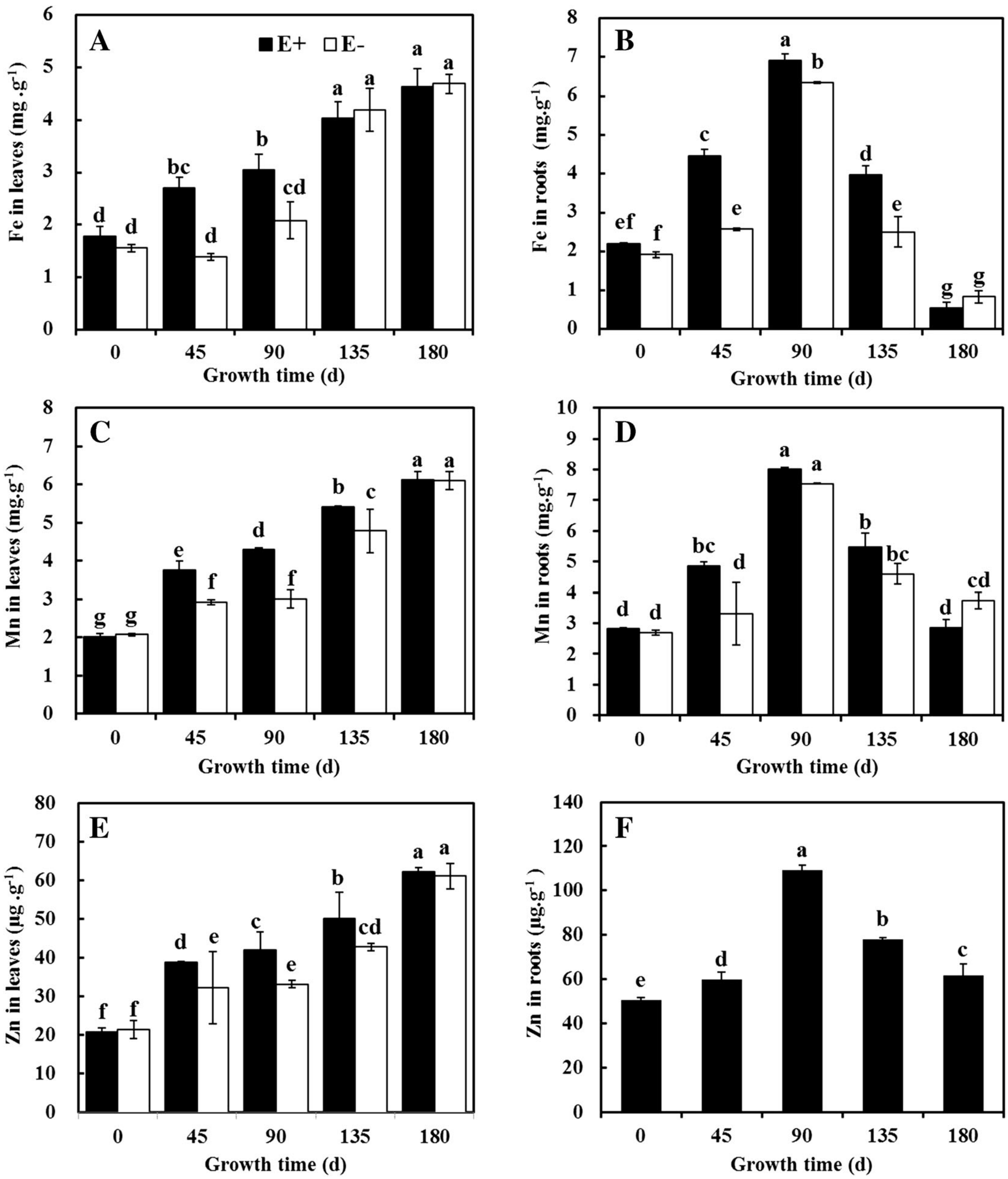

Fig. 9 Fe and Mn contents of leaves and roots, and the Zn content of leaves of *Lolium perenne* plants with (E+) and without (E-) *Epichloë* endophyte at different growth times (A, B, C, D and E), and Zn content of roots of plants at different growth times

photosynthesis, the sugar, water and nutrition transfer, protein and starch synthesis, stomatal movement, N and organic acid metabolism, and stress tolerance (Besford, 1978; Wang et al., 2013). An *Epichloë* endophyte was previously observed to affect the K content (Rahman and Saiga, 2005). Malinowski et al. (1998a) showed that the chemistry of the rhizosphere may be influenced by endophyte infection in different ways, depending on host plant genotype and environmental factors (nutrient availability). Results from the present study (Fig.7C and D) support these finding showing that *Epichloë* endophyte infection increased the K content in leaves and roots at the early stage of plant growth. In addition, the K content in leaves and roots not only has positive and direct effect on plant survival, but also positive indirect effects on plant survival by directly negatively affecting

Table 5 Two-way ANOVA for the effects of endophyte status (E) and growth time (T) on the Mn, Fe, Zn and Cu contents of leaves and roots of *Lolium perenne*

Source	df	Mn content				Fe content				Zn content				Cu content			
		leaves		roots		leaves		roots		leaves		roots		leaves		roots	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
E	1	80.7	<0.001	1.0	0.327	8.0	0.032	71.5	<0.001	0.2	0.684	0.2	1.235	5.0	0.035	17.0	<0.001
T	4	384.0	<0.001	51.3	<0.001	49.3	<0.001	449.7	<0.001	92.3	<0.001	23.3	<0.001	165.1	<0.001	221.5	<0.001
E x T	1	24.3	<0.001	3.2	0.045	3.1	0.048	18.2	<0.001	15.4	<0.001	1.2	0.781	16.5	0.006	1.7	0.976

the amount of Ca, Mg, Mn and Cu in leaves, Ca in roots, dry weights of leaves. And further, the endophyte has positive and direct influence on root metabolic activity, and direct negative affect on the Cu content of roots. It is possible that K content in leaves and roots was the main factor to affect plant survival rate in the indirect action of endophytes because K is involved in many metabolic processes and synthesis of compounds.

Mn is an essential microelement in plants that has direct participation in photosynthesis, promotion of N metabolism, regulation of redox status, increase in respiratory intensity and promotion of the hydrolysis of carbohydrates (Lanquar et al., 2010; Sieprawska et al., 2016). Manganese deficiency in plants leaves leads to accumulation of free amino acids, and reduced protein synthesis (Labanauskas and Handy, 1970). Organic matter had different effects on the conversion and availability Mn in soil by affecting redox process of Mn and promotion of the dissolution of Mn via chelation (Sanchez and Kamprath, 1959). Under Mn deficiency, plants show decreased nitrate reductase activity and increased concentration of nitrate nitrogen (Heenan and Campbell, 1980; Gong et al., 2011). P can aggravate Mn deficiency in plants or induce plants to absorb more manganese (Pai et al., 2011). *Epichloë* infection increased the Mn content of host plants (Zabalgogeazcoa et al., 2013). Our study showed that *Epichloë* endophyte infection had indirect and positive effects on Mn content of leaves by affecting OC, N, P, K, Ca and Mg content.

Conclusions

In this work we examined the effects to *Epichloë* endophyte infection on perennial ryegrass survival rate, plant growth, root metabolic activity, and nutrient content under low fertility conditions. The presence of the *Epichloë* endophyte relieved the withering and yellowing of plants in the short term (0 to 90d). The possible mechanisms by which *E. festucae* var. *lolii* infection enhances survival rate of plants include improved root metabolic activity, increased biomass production, and increased concentration of other nutrient elements. *Epichloë* endophyte had significant direct or indirect effects on plant survival rate, K content in leaves and roots, Mn content of leaves, and the activity and dry weight of roots. These findings also confirmed that the *Epichloë* endophyte effect was dependent on

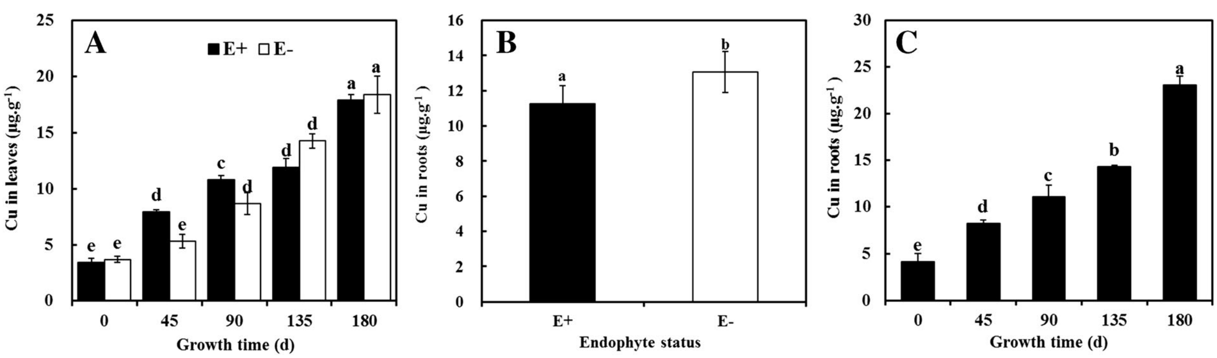

Fig. 10 Cu content in leaves of *Lolium perenne* plants with (E+) and without (E-) *Epichloë* endophyte at different growth times (A), and the Cu content in roots of E+ and E- plants (B), and at different growth times (C)

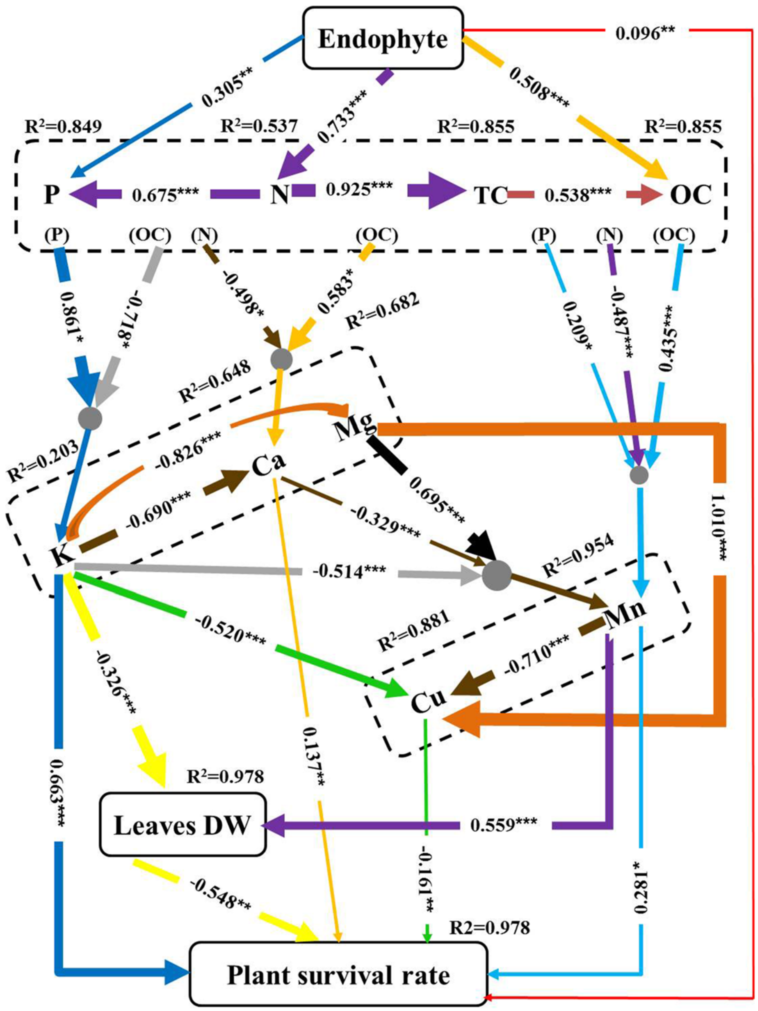

Fig. 11 Structural equation model of base on leaves data. The model depicts direct and indirect effects of endophyte on response variables [C, N, P, contents of leaves (OC, TC, N, and P), K, Ca, and Mg, the trace elements of leaves (Mn and Cu), dry weights in leaves and plant survival rates]. To facilitate interpretation, response variables of a similar type are grounded within bold-dashed boxes (such as OC, TC, N, and P of leaves). In order to avoid excessive intersecting lines, (OC, TC, N, and P) and related response variables are linked by arrows one by one, and the interaction effects of upper-level response variables are depicted with arrows that point to a solid grey dot, and the dot points to the lower level of response variables. Arrows of different colors show different paths of influence of endophyte on plant survival rate. Numbers on arrows are standardized path coefficients (SPC), indicating the strength of the relationship (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). The width of arrows is proportional to the magnitude of the values. The numbers (R^2) on the top of the response variables represent the proportion of explained variance. Results of model fitting: $\chi^2 = 28.099$, df = 36, $P = 0.824$

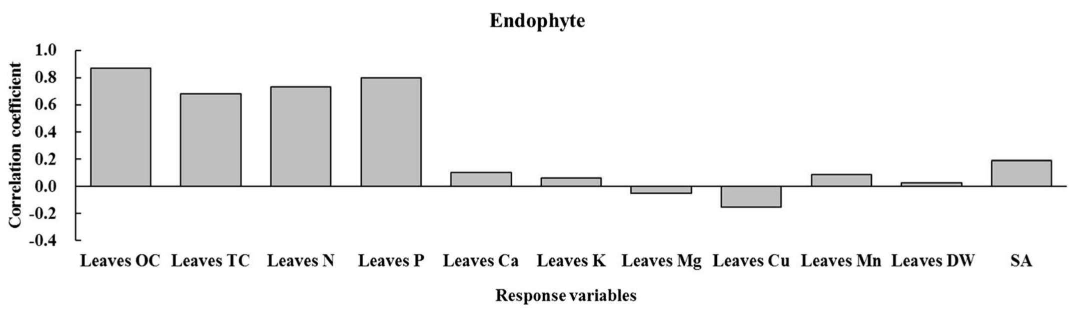

Fig. 12 Standardized total effects of endophyte infection on response variables of leaves in the SEM model

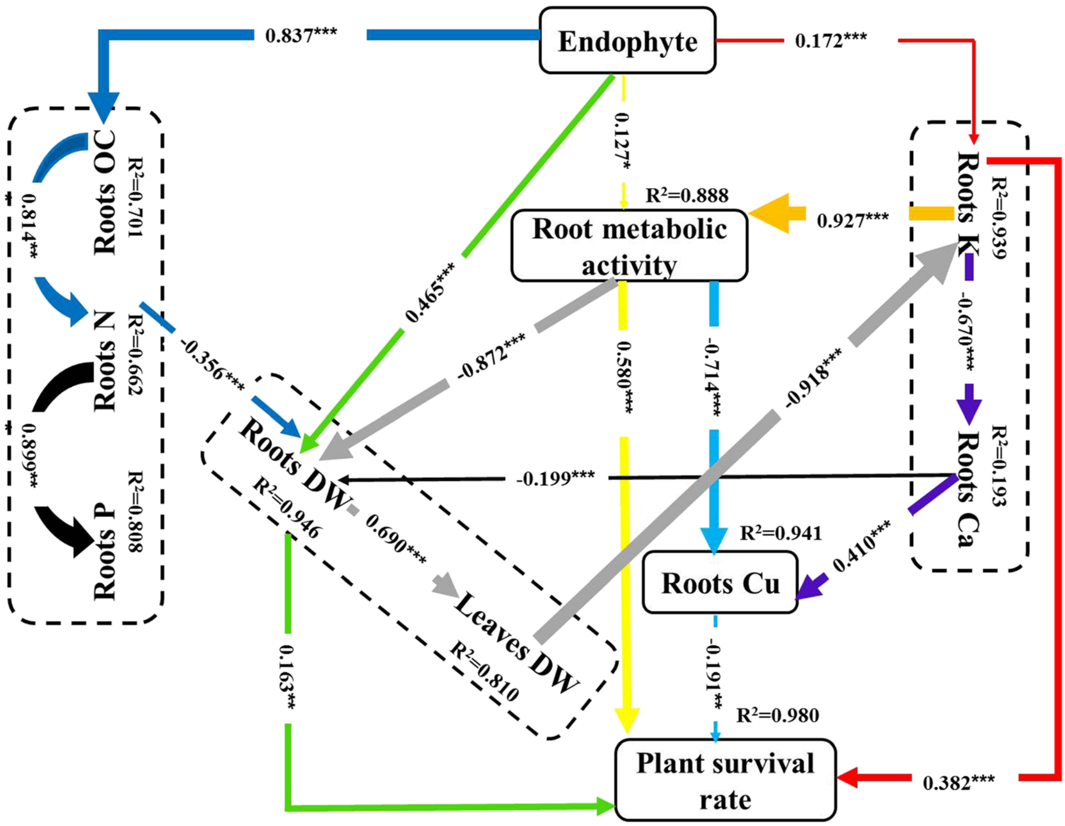

Fig. 13 The structural equation model based on the data from leaves and roots. The model depicts direct and indirect effects of endophyte on response variables [C, N, P of roots (OC, N, and P) contents, K and Ca contents of roots, Cu content of roots, root metabolic activity, dry weight of leaves and roots, and plant survival rate]. To facilitate interpretation, response variables of a similar type are grounded within bold-dashed boxes (such as OC, TC, N, and P of roots). The arrows of different color show different paths of influence of endophyte on plant survival rate. Numbers on arrows are standardized path coefficients, indicating the strength of the relationship (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). The width of arrows is proportional to the magnitude of the values. The numbers (R^2) on the top of the response variables represent the proportion of explained variance. Results of model fitting: $\chi^2 = 35.988$, df = 36, $P = 0.469$

plant growth time. One factor affecting whether endophytes have positive or negative effects on the host is soil nutrient availability. Under low nutrient availability negative effects are exacerbated as plants grow and nutrients become more limited. Our results have both theoretical and practical significance. The presence of *E. festucae* var. *lolii* increased tolerance of *L. perenne* to low nutrients, and we propose that grasses infected by *Epichloë* endophytes could be used to remediate degraded lands, or ameliorate barren soils as pioneer plants.

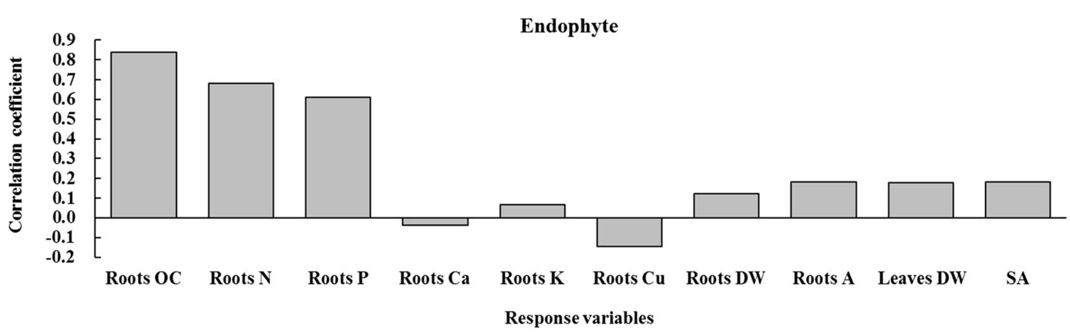

Fig. 14 Standardized total effects of endophyte infection on response variables of leaves and roots in the SEM model

Funding information This research was supported by the National Basic Research Program of China (2014CB138702), the Second Tibetan Plateau Scientific Expedition and Research (STEP) program (2019QZKK0302), Program for Changjiang Scholars and Innovative Research Team in University, China (IRT17R50), Fundamental Research Funds for the Central Universities (LZUJBKY -2020-kb10 and it11) and 111 Project (B12002). The authors are thankful for support from USDA-NIFA Multistate Project W4147, and the New Jersey Agricultural Experiment Station.

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