

# Response of *Neotyphodium lolii*-infected perennial ryegrass to phosphorus deficiency

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## ABSTRACT

It has been demonstrated that endophyte-infected (EI) ryegrass performed better in response to N deficiency than its endophyte-free (EF) counterpart. When P is considered, there is a lack of related information. In this study, *Lolium perenne* L. infected with *Neotyphodium lolii* was employed to establish EI and EF populations. Soil-grown EI and EF ryegrass were tested for their responses to P deficiency. The results showed that the endophyte infection improved the adaptability of ryegrass to P deficiency. When P was limited, EI roots were significantly longer (EI, 398.8; EF, 323.4 m/pot) and heavier (EI, 30.58; EF, 23.20 g/pot) than EF roots; the root: shoot ratio of EI plants was greater than that of EF plants ( $P < 0.05$ ). The content of total phenolics and organic acids was significantly greater for EI roots than for EF roots at low P supply; the concentration of both, however, was not improved by the endophyte infection. This suggested that it was the higher root dry weight (DW) that contributed to the higher content of total phenolics and organic acids for EI plants, and the endophyte infection might have negligible effects on chemical modification of perennial ryegrass. Endophyte infection did not increase P uptake rate but did significantly improve P use efficiency of ryegrass in response to P deficiency (EI, 0.734; EF, 0.622 g DW/mg P).

**Keywords:** endophyte; phosphorus deficiency; total phenolics; organic acids; acid phosphatase activity; *Lolium perenne* L.

*Neotyphodium* endophytes and cool-season grasses are mutually symbiotic. On one hand, the grasses provide photosynthates for the fungi; on the other hand, the endophytes can enhance the hosts' growth and protect them from biotic and abiotic stresses, contributing thus to their adaptability. Abiotic attributes affected by *Neotyphodium* endophytes include drought resistance (Hesse et al. 2003), light stress (Lewis 2004), high temperature (Marks and Clay 1996), mineral stress, etc. Studies on endophyte-related responses of grasses to nutrient acquisition focused on the influence of nitrogen, since this element is a constituent of alkaloids in infected plants. Arechavaleta et al. (1989) found beneficial effects of the endophyte infection in tall fescue and perennial ryegrass only at high N concentrations; Cheplick et al. (1989) hypothesized that endophyte might impose an additional metabolic cost on the host grass, especially

under nutrient limited conditions, since endophyte might compete with the host for nutrient elements. Subsequently, Belesky et al. (1991) reported that the endophyte infection did not affect carbohydrate accumulation in tall fescue at low N supply; therefore, they thought that the endophyte infection was not detrimental to the host plant even under nutrient limited conditions. In perennial ryegrass, Lewis (2004) reported that EI grass used N more effectively than EF grass when grown at low N availability. Other studies with perennial ryegrass, however, showed minimal or inconsistent effects of endophyte at low N concentrations (Durand et al. 2002). This suggests a specificity of endophyte-host grass associations in terms of N use efficiency.

Similar to N nutrition, P availability influenced ergot alkaloid production in EI grasses (Malinowski et al. 1999a). Reports of endophyte-related re-

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sponses of grasses to P nutrition are rather limited. Azevedo and Welty (1995) firstly confirmed reduced photosynthesis rates and growth of EI tall fescue at high P availability, and it was also found that high concentrations of inorganic P in endophyte hyphae grows epiphytically on tall fescue roots, similar to that found in mycorrhizal fungi. Malinowski et al. (1999b) found that EI tall fescue expressed increased root absorption area through reduced root diameter and increased root hair length when compared with the EF counterpart. The rhizosphere of tall fescue was also chemically modified in response to endophyte infection resulting from the release of phenolic-like compounds from roots (Malinowski et al. 1998). Phenolic compounds released from EI roots had the ability to reduce Fe outside of the root system and chelate Cu in nutrient solution in tall fescue (Malinowski et al. 2004). The known results suggest that root modification as well as accumulation of phenolic compounds in response to the infection with *Neotyphodium* endophyte may be two different modes to overcome P deficiency in tall fescue.

Similar to tall fescue, perennial ryegrass is commonly associated with *Neotyphodium* endophytes. However, it is not known what the attribute of *Neotyphodium* endophyte is like under P-deficiency stress for perennial ryegrass-*Neotyphodium* endophyte association. Up to now, no related research results have been published. Previously we found that hydroponically-grown EI ryegrass grew better, and at the same time accumulated more phenolic compounds, than EF ryegrass under P-deficiency stress. In this context soil-grown ryegrass was examined in order to find the beneficial function of endophyte in ryegrass to overcome P deficiency.

## MATERIAL AND METHODS

Seeds from the same plants of EI ryegrass (*Lolium perenne* L. cv. SR4000) (originally infected with *Neotyphodium lolii*, bought from the Beijing Clover Seed Company, China) were treated in two different ways: some were placed on moist filter paper and germinated at 25°C to get EI plants, others were exposed to a heat treatment in the bath (43°C 15 min, then 57°C 25 min) and then placed on moist filter paper to get EF plants. EI and EF plants used in this experiment were reared in spring in 1999, multiplied and selected for uniformity in spring in 2000, 2001 and 2002.

The endophyte status was checked in each plant before the experiment. In June 2002, EI and EF plants (13 ramets per pot) were transplanted into plastic pots (28 cm in diameter and 25 cm in depth) filled with 12.5 kg of sandy soil. The field capacity and permanent wilting point of the soil were 25.4 and 5.3 g water per 100 g oven-dry soil, respectively. The untreated soil had 1.05% organic substance, 270 mg/kg total N, 3.1 mg/kg extractable P, and 9.2 mg/kg K.

The experiment was carried out at the campus experiment site of the Nankai University, Tianjin, China, in 2002. There were two periods of P stress treatment: the first period was between 15 July and 14 September, and the second one between 30 September and 30 October. The first harvest was taken on 15 September, leaving a stubble height of about 5 cm. Two-factor randomized-block design was used. The first factor was P treatment and 2 levels of P treatments were imposed, i.e. P supply (P+) and P deficiency (P-), the other factor was endophyte status, i.e. EI and EF. Each treatment was replicated five times. P+ treatment was achieved by the addition of P to the soil in the form of the Hoagland nutrient solution. The composition of the nutrient solution was ( $\mu\text{M/l}$ ):  $\text{Ca}(\text{NO}_3)_2$  5000,  $\text{KNO}_3$  5000,  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  2500,  $\text{KH}_2\text{PO}_4$  2000,  $\text{Na}_2\text{C}_{10}\text{H}_{14}\text{O}_3\text{N}_2$  29,  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  20,  $\text{H}_3\text{BO}_3$  45,  $\text{MnSO}_4$  6.6,  $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$  0.8,  $\text{H}_2\text{MoO}_4$  0.6,  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  0.4. For P- treatment, 2000  $\mu\text{M/l}$  KCl was added instead of  $\text{KH}_2\text{PO}_4$ . Two liters of nutrient solution per pot were added once a month, and four times in total.

During the experiment, two main stems per pot were selected randomly and the length and width of each leaf on them were measured every three days with a ruler. The increase in tiller number per pot was recorded every seven days. By the end of the experiment, leaves, sheaths and roots were harvested separately. Root length was measured with a root length scanner (CI-203). P concentration in plant tissues was analyzed using the method of Bao (2000). The activity of acid phosphatase (ACP) and the content of organic acids (OA) were analyzed using the method of Shen et al. (2001). Total phenolics were analyzed according to the method of Malinowski et al. (1998). Plants were dried at 60°C for three days to record leaf, sheath and root DW. P use effectiveness was determined based on total plant DW. All data were analyzed using one-way and two-way analyses of variance (ANOVA) using programs of the SPSS statistical package.

Table 1. Analysis of variance summary of soil P level (P) and endophyte status (E) upon growth parameters of perennial ryegrass

Source	LAR	Tiller No.	Tiller weight	Leaf DW		Sheath DW	Root DW	Root/Shoot ratio	Root length
				1	2				
P	NS	NS	NS	*	*	NS	NS	NS	NS
E	NS	NS	NS	NS	NS	NS	*	*	*
P × E	NS	NS	NS	NS	NS	NS	NS	NS	NS

\*significant at  $P < 0.05$ ; NS – not significant, LAR – leaf area

## RESULTS

### Developmental characteristics

Most of the measured plant growth parameters were influenced by neither P availability in the soil nor endophyte status (Table 1). The interaction of P source with the endophyte status had no significant effect on either of measured growth parameters. The effect of P level was significant only for leaf DW in a certain experimental period. Total leaf DW, however, was not influenced by P level because it was changed in different trend between two experimental periods. As for the endophyte status, it significantly influenced root growth of perennial ryegrass. In response to P deficiency, when compared with EF partners, EI plants showed higher root DW (EI, 30.58; EF, 23.20 g/pot), bigger root: shoot ratio (EI, 0.82; EF, 0.62) and greater total DW (EI, 68.92; EF, 61.73 g/pot). At the same time, root length of EI plants was not affected while that of EF plants was greatly reduced. Therefore, EI plants had longer root system (Figure 1). These results suggested that the endophyte infection was beneficial to the root development of perennial ryegrass only at low P availability.

### Content of total phenolics and organic acids and acid phosphatase activity

Both total phenolic content and OA content in roots of ryegrass were significantly influenced by P level as well as by the interaction of P source with the endophyte status (Table 2); in leaves they were both affected neither by P level nor by endophyte status. In response to P deficiency, total phenolic content increased in roots but kept unchanged in sheaths and leaves of ryegrass, regardless of the endophyte status. Endophyte infection helped the host grass to accumulate more phenolic compounds in roots when P was limited (Figure 2), but had no beneficial effect on the concentration of phenolics (EI, 0.0086; EF, 0.0081% DW). As for sheaths and leaves, their total phenolic content was hardly affected by the endophyte infection.

Similar to the total phenolic content, OA content in roots was greatly affected by P level in the soil but the same trend did not appear in leaves. At high P level, EI roots tended to have less OA than EF roots; at P deficiency, OA content in EI roots was significantly higher than that in EF roots (Figure 3). However, OA concentration in EI and EF roots was similar, which was consistent with the concentration of total phenolics. The phe-

Table 2. Analysis of variance summary of soil P level (P) and endophyte status (E) on the content of total phenolics and organic acids and acid phosphatase activity of perennial ryegrass

Source	Total phenolic content			Content of organic acids		Acid phosphatase activity	
	roots	sheaths	leaves	roots	leaves	roots	leaves
P	*	NS	NS	*	NS	NS	NS
E	NS	NS	NS	NS	NS	NS	NS
P × E	*	NS	NS	*	*	*	NS

\*significant at  $P < 0.05$ ; NS – not significant

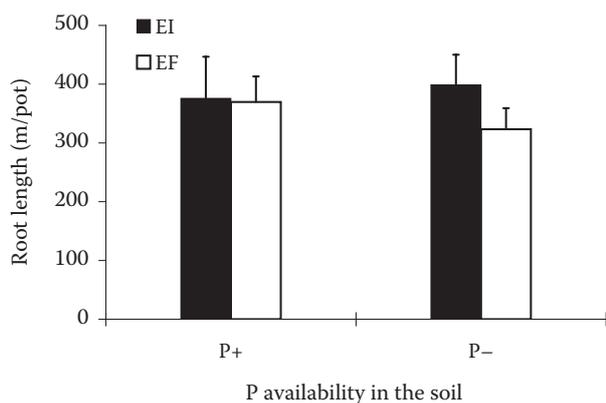


Figure 1. Root length of endophyte-infected (EI) and endophyte-free (EF) perennial ryegrass grown in P deficient (P-) and P supply (P+) soils; bars indicate 1SE

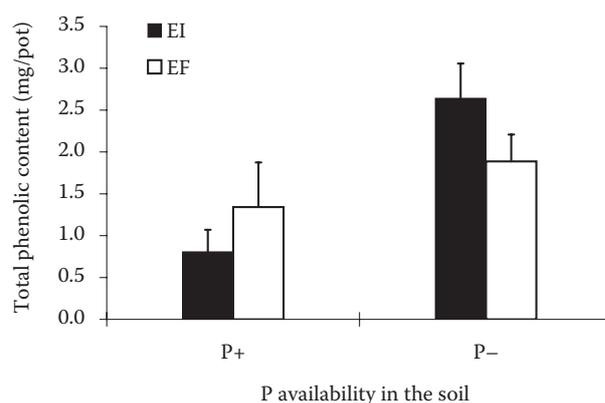


Figure 2. Total phenolic content for roots of endophyte-infected (EI) and endophyte-free (EF) perennial ryegrass grown in P deficient (P-) and P supply (P+) soils; bars indicate 1SE

nomenon resulted from EI plants having higher root DW than their EF counterparts.

In this study, leaves and roots of ryegrass all showed ACP activity. Neither P level nor endophyte status individually influenced the ACP activity in different organs of ryegrass. However, the interaction of P source with the endophyte status significantly affected the ACP activity in roots (Table 2). In response to P deficiency, ACP activity in EI roots significantly increased while that in EF roots kept unchanged; therefore the ACP activity in EI roots was significantly higher than in EF roots (Figure 4).

### Phosphorus acquisition

P concentration in different organs was positively related to P source (Tables 3 and 4). All plant organs

accumulated significantly more P at P supply level than at P deficiency, regardless of the endophyte status. Endophyte infection significantly affected P concentration in sheaths and leaves, and the effect was different within different plant organs and different P levels. At P supply, EI leaves had more P than EF leaves, but P concentrations in roots and sheaths were similar in EI and EF plants. At P deficiency, EI sheaths had less P than EF ones, while P concentration in roots of both was similar. When root DW was considered, P content in EI roots was higher than that of EF roots because of larger root biomass for EI roots.

P uptake rate (mg/day/pot) was significantly influenced by P level in the soil, but not related to the endophyte status and interaction of the endophyte infection with P level (Tables 3 and 4). P uptake rate was 2.1 times higher for EI plants and 1.9 times higher for EF plants grown at P supply

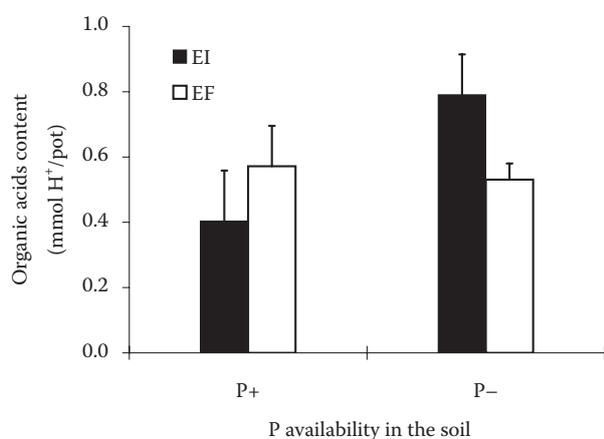


Figure 3. Organic acids content for roots of endophyte-infected (EI) and endophyte-free (EF) perennial ryegrass grown in P deficient (P-) and P supply (P+) soils; bars indicate 1SE

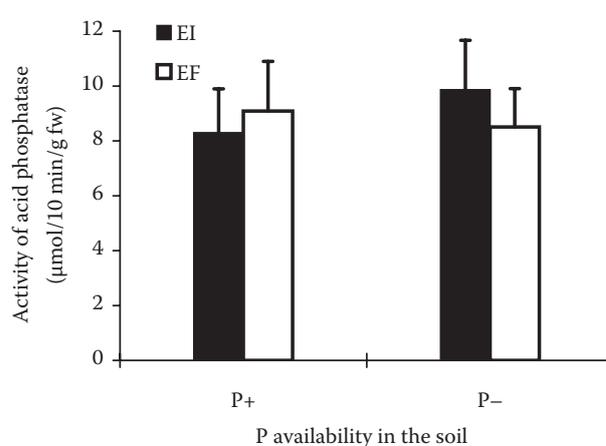


Figure 4. Acid phosphatase activity for roots of endophyte-infected (EI) and endophyte-free (EF) perennial ryegrass grown in P deficient (P-) and P supply (P+) soils; bars indicate 1SE

Table 3. Analysis of variance summary of soil P level (P) and endophyte status (E) on P concentration, P uptake rate and P use efficiency of perennial ryegrass

Source	Root P	Sheath P	Leaf P	Total P	P uptake rate	P use efficiency
P	*	*	*	*	*	*
E	NS	*	*	NS	NS	*
P × E	NS	NS	*	NS	NS	*

\*significant at  $P < 0.05$ ; NS – not significant

than at P deficiency. Endophyte infection did not improve P uptake rate of the host grass grown in either level of P source.

P use efficiency (g DW/mg P) was significantly influenced by P level in the soil, the endophyte status as well as the interaction of the endophyte infection with P level (Tables 3 and 4). Generally, P use efficiency was greater for plants grown at P deficiency than at P supply. In response to P deficiency, P use efficiency of EI plant increased 2.1 times while that of EF plants increased 1.7 times. Endophyte infection improved P use efficiency, but the beneficial effect was only significant at P deficiency.

## DISCUSSION

The endophyte infection did not improve the shoot growth of perennial ryegrass in the long run. Root development, however, was significantly improved by the endophyte infection, which was demonstrated by the fact that root DW and length of EI plants were significantly greater than those of EF plants in response to P deficiency. Similar results were also achieved in tall fescue. In series of reports made by Malinowski et al. (1998, 1999a, b, 2000), they found that root DW of specific EI genotype (DN4) was 10% greater than that of EF plants (Malinowski and Belesky 1999, 2000); EI plants

produced roots with smaller diameters and longer root hairs (Malinowski et al. 1998, 1999b). Roots are responsible for mineral acquisition. The increase of either root weight or root length will enhance total root surface area that functions in mineral acquisition. Thus, the endophyte infection may help the host grass under low P conditions by improving its root development.

Except alterations in root morphology, another mechanism of mineral uptake, an activity of root exudates, was demonstrated in tall fescue in response to P deficiency (Malinowski et al. 2000). As for perennial ryegrass, total phenolic content, but not its concentration in EI roots, was greater than at EF roots only under P-deficient conditions, which disagreed with observations in tall fescue (Malinowski et al. 1998). In tall fescue, Malinowski et al. (1998) reported that the concentration of total phenolics in roots for EI tall fescue was greater than that for EF plants when subjected to P deficient stress. Ryegrass roots also released phenolic compounds in response to P deficiency, but this process seemed to be dependent on root DW and independent of endophyte infection.

Except for phenolics, other root exudates such as OA can mobilize P from the soil solid-phase. OA can acidify the rhizosphere and alter anion exchange, thereby increasing the solubility of P. Numerous researches documented that P-defi-

Table 4. P concentration (%), P uptake rate (mg/day/pot) and P use efficiency (g DW/mg P) of endophyte-infected (EI) and endophyte-free (EF) perennial ryegrass grown in P deficient (P-) and P supply (P+) soils

P level	Endophyte status	Root P	Sheath P	Leaf P	P uptake rate	P use efficiency
P+	EI	0.210 a	0.315 a	0.414 a	1.540 a	0.357 c
	EF	0.211 a	0.339 a	0.339 b	1.412 a	0.364 c
P-	EI	0.121 b	0.140 c	0.186 c	0.744 b	0.734 a
	EF	0.125 b	0.182 b	0.205 c	0.765 b	0.622 b

Within rows, the same letter denotes non-significant difference, while different letters denote a significant difference ( $P = 0.05$ )

ciency-resistant plants excreted large amounts of OA (Neumann et al. 1999), which helped to increase the availability of mineral bound phosphates and the release of phosphates from humic substances. Up to now, no published research work concentrated on whether production of OA was involved in grass-endophyte associations in response to P deficiency. In this study, EI roots produced larger amounts of OA than EF roots, but the concentration of OA in both was similar, which agreed with the concentration of total phenolics. The content of both phenolics and OA was greater for EI plants than for EF plants only because EI plants had higher root DW, which suggested that the endophyte infection may have neglectable effects on chemical modification of ryegrass roots. Malinowski et al. (2004) also found that the Cu<sup>2+</sup>-binding activity by root exudates of perennial ryegrass was not affected by the endophyte infection; nevertheless, similar to tall fescue, roots of perennial ryegrass release exudates with Cu<sup>2+</sup>-binding activity in response to P-deficiency stress in nutrient solution. Root exudates of perennial ryegrass may be different in nature from those of tall fescue.

ACP is excreted together with the exudation of OA (Neumann et al. 1999). At the same time, plant uptake of P hydrolyzed by ACP is thought to be improved by the presence of citrate. ACP can release inorganic phosphate from organophosphates. The production and excretion of ACP is considered to be one component of a plant phosphate-starvation rescue system (Wasaki et al. 2003). It was found that ACP activity increased more in roots of VAM bean and endomycorrhizal maize growing under P stress than in noncolonized controls (Fries et al. 1998), in which an increase of the ACP activity was dependent not only on fungal biomass, but also on soil P availability. In grass-endophyte associations, no related work was available. In our study, the ACP activity was greater in EI roots than in EF roots in response to P deficiency, which agreed with Shen et al. (2001) in other endophyte-unrelated plants. As for endophyte-related grass, this is the first report. According to Duff et al. (1989), a higher intracellular ACP activity may allow for a more efficient utilization of P in primary metabolism.

In our study, both P uptake rate and P concentration in different plant organs were significantly influenced by P level, but not influenced by the endophyte status. Total P content of EI roots, however, was significantly greater than that of EF roots because EI had larger root DW in re-

sponse to P deficiency. Unlike P uptake rate, P use efficiency was greatly improved by the endophyte infection when P was limited, which might be related to a higher ACP activity in EI plants; this higher ACP activity might enable ryegrass to reuse limited P source, which would be further beneficial to the development of ryegrass roots.

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