

# Shoot specific fungal endophytes alter soil phosphorus (P) fractions and potential acid phosphatase activity but do not increase P uptake in tall fescue

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## Abstract

**Aims** An experiment was performed to test how different fungal endophyte strains influenced tall fescue's ability to access P from four P sources varying in solubility.

**Methods** Novel endophyte infected (AR542E+ or AR584E+), common toxic endophyte infected (CTE+), or endophyte-free (E-) tall fescues were grown for 90 days in acidic soils amended with 30 mg kg<sup>-1</sup> P of potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), iron phosphate (FePO<sub>4</sub>), aluminum phosphate (AlPO<sub>4</sub>), or tricalcium phosphate ((Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>), respectively.

**Results** Phosphorus form strongly influenced plant biomass, P acquisition, agronomic P use efficiency, microbial communities, P fractions. P uptake and vegetative biomass were similar for plants grown with AlPO<sub>4</sub>, Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, and KH<sub>2</sub>PO<sub>4</sub> but greater than in control and FePO<sub>4</sub> soils. Infection with AR542E+ resulted in significantly less shoot biomass than CTE+ and E-

varieties; there was no influence of endophyte on root biomass. The biomarker for arbuscular mycorrhizal fungi (AM fungi, 16:1ω5c) was selected as an effective predictor of variations in P uptake and tall fescue biomass. Potential acid phosphatase activity was strongly influenced by endophyte × P form interaction.

**Conclusions** Endophyte infection in tall fescue significantly affected the NaOH-extractable inorganic P fraction, but had little detectable influence on soil microbial community structure, root biomass, or P uptake.

**Keywords** *Neotyphodium coenophialum* · *Epichloë coenophiala* · Microbial community · Phospholipid fatty acids (PLFAs) · Phosphorus fractions · Potential acid phosphatase activity (AcPase)

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## Introduction

Compared with the other major nutrients, phosphorus (P) is by far the least available to plants in most soil conditions (Hinsinger 2001), because it easily forms insoluble complexes with Fe and Al in acidic soils and Ca in calcareous or alkaline soils (Bertrand et al. 2003; Akhtar et al. 2009). Replenishment of soil P reserves through fertilization is common, but the long-term sustainability of this practice is in question, because only 50 % of economically recoverable P reserves are estimated to remain by the middle of this century (Abelson 1999; Lynch and Brown 2008; Akhtar et al. 2009). As such, there is an urgent need to explore and deploy alternative strategies enhancing P-acquisition and use

efficiency in soils, especially in P-limiting conditions (Akhtar et al. 2009).

Tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh) infected with the common toxic shoot-specific fungal endophyte *Epichloë coenophiala* (formerly *Neotyphodium coenophialum*) demonstrates a competitive advantage over endophyte-free grasses when grown with limited nutrient resources, especially P (Marks et al. 1991; Bacon and Hill 1996). Endophyte-related adaptations involved in enhancing P uptake include increased root surface area and root hair number, which have been well documented in many growth conditions including agar (Ding et al. 2015), hydroponics, and soils (Malinowski et al. 1998a, b, 1999). Besides remodeling of root system architecture, the discovery of endophyte-influenced chemical modifications in the rhizosphere of tall fescue, which are postulated to influence the uptake of certain P-bearing minerals, has deepened our understanding of endophyte involvement in mineral nutrition (Malinowski et al. 1998a, 1999; Malinowski and Belesky 2000).

Common toxic endophyte infected tall fescue produces ergot alkaloids that are toxic to grazing livestock creating a condition called ‘fescue toxicosis’ resulting in significant losses for the livestock industry. Because of fescue toxicosis, efforts have been made to replace the common toxic endophyte strain (CTE +) with so-called “novel” non-mammal-toxic strains (e.g., AR542, AR584). These novel endophyte strains retain the non-toxic class of alkaloids (i.e., lolines and paramine) which are beneficial to the plant while producing minimal or none of the alkaloids (i.e., ergot alkaloids) detrimental to grazing animals (Hunt and Newman 2005). We recently showed that novel endophyte genotype and tall fescue cultivar interact to differentially effect the composition of root exudates; some of which were identified to play a role in P acquisition (Guo et al. 2015). The result suggests that alteration of rhizodeposits in response to nutrient stress and endophyte genotype may act together to overcome P deficiency in tall fescue. However, there are currently no studies addressing how the novel endophytes influence P mobilization from sparingly soluble P forms.

To address these questions we conducted a greenhouse study in which we grew tall fescue either without endophyte infection, or infected with the common toxic endophyte, or one of two different novel endophytes for 90 days in low P acidic soils spiked with four P sources of varying solubility. Our interest was to determine if

endophyte strain influenced P acquisition from sparingly soluble forms, how novel endophytes compared to the common toxic strains, and what rhizosphere processes are involved in any of the differences observed. Based on our previous work and that of others, we hypothesize that endophyte infected grasses would be better able to access P from sparingly soluble P forms, uptake and plant biomass production would vary across endophyte strain, and these differences would be linked to differences rhizosphere microbial community structure and function.

## Materials and methods

### Soil properties

A Sadler silt loam soil (0–20 cm in depth) was collected from a fallow (60 years) field dominated by mixed grasses and weeds at the University of Kentucky Research and Education Center, Princeton, KY, USA. The soils were air-dried at room temperature in the greenhouse for 72 h and ground to pass through a 2-mm sieve. Samples were sent to the University of Kentucky Regulatory Services Soil Testing Laboratory for analysis of basic soil properties (<http://soils.rs.uky.edu/tests/methods.php>). Soil pH was found to be 5.50, organic matter content 3.2 %, total N 0.17 %, and concentrations ( $\text{mg kg}^{-1}$ ) of Mehlich III extractable elements were: 10.3 (P), 72.8 (K), 1010 (Fe), 103 (Mg), 1.7 (Zn), 118 (Al), and 803 (Ca).

### Plant growth conditions, plant and soil sampling

The pot experiment was set up in a completely randomized design with 4 replicates per treatment [endophyte (4) x P treatment (5)] for a total of 80 pots. Pots were prepared by combining 400 g of soil with the P treatment and basic fertilizer nutrients in 1 liter plastic bags, mixing thoroughly, and then transferring the contents to 500 ml round plastic pots. Each pot had 2 layers of polyethylene mesh lining the bottom to prevent soil loss. P treatments were imposed by adding  $30 \text{ mg P kg}^{-1}$  of the following P forms: potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ , K-Ps), iron phosphate ( $\text{FePO}_4$ , Fe-Ps), aluminum phosphate ( $\text{AlPO}_4$ , Al-Ps), and tricalcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ , Ca-Ps). Soils receiving no additional P served as a control. The concentration of P added ( $30 \text{ mg kg}^{-1}$ ) together with the native soil P

(~10 mg kg<sup>-1</sup>) represents a medium fertilizer rate for acidic soils (Wright et al. 1987). Additional macro and micro nutrients were supplied as follows: 125 mg K kg<sup>-1</sup> soil (as KCl), 75 mg N kg<sup>-1</sup> soil (as NH<sub>4</sub>NO<sub>3</sub>), 0.1 µg Mo kg<sup>-1</sup> (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> · 4H<sub>2</sub>O), 1 µg B kg<sup>-1</sup> (H<sub>3</sub>BO<sub>3</sub>), 5 µg Cu kg<sup>-1</sup> (CuSO<sub>4</sub> · 5H<sub>2</sub>O), and 5 µg Zn kg<sup>-1</sup> (ZnSO<sub>4</sub> · 7H<sub>2</sub>O). Pots with soil only were then maintained at 60 % water holding capacity (WHC) using double deionized water (DDI; MilliQ) by weight for 2 weeks. Soils from four replicates of each P treatment and the control were collected to determine the initial total and sequential P fractions prior to plant growth, which were treated as pre-planted soils.

Tall fescue cultivar PDF seeds either uninfected with *E. coenophiala* (E-), or infected with common toxic endophyte (CTE+), or one of two novel endophytes (AR542E+ or AR584E+) were obtained from the Samuel Roberts Noble Foundation (Ardmore, OK, USA). To maintain endophyte infection rates, seeds were stored under dry, cool and dark conditions until planting. Endophyte infection rates were determined after 4 weeks of plant growth using an Agronostics Phytoscreen immunoblot test kit (Hiatt et al. 1999). Using this methodology, infection rates for CTE+, AR542E+, AR584E+ and E- seeds used in this study were 75.0, 84.4, 96.8 and 6.0 %, respectively. Sixty seeds of each combination were uniformly sown in their respective pots. Pots were randomly arranged on a greenhouse bench at a University of Kentucky greenhouse facility (Lexington, USA) each receiving 14 h of light per day with day/night temperatures of 25/15 °C. Plants were thinned to 45 per pot after seedling emergence, and grown for a total of 90 days. Moisture content was maintained at 60 % of WHC by adjusting each pot to weight daily with deionized water. Additional N was applied weekly as NH<sub>4</sub>NO<sub>3</sub> solution at 4.8 mg N pot<sup>-1</sup>. After 90 days of growth the plants had developed a dense root system that explored the entirety of the pot, thus we assumed that all the soil in the pot was under the direct influence of the plant and could be considered rhizosphere soils. Soils were collected by removing the plants from each pot and vigorously shaking the root system over a bench pad. Fine roots were meticulously removed from the rhizosphere soil before it was transferred into Whirl-Pak® (Nasco, Fort Watkins, Wisconsin) bags and immediately shock-frozen in liquid nitrogen and stored at -20 °C until preparation for analysis.

Plant biomass, P content and agronomic P use efficiency (APE)

After washing the plant roots, the shoots were separated from the roots at the crown, the tillers counted and roots and shoots placed in separate brown paper bags for drying at 65 °C until a constant weight was achieved. After drying, root and shoot tissue samples were dry-ashed at 500 °C in a muffle furnace for 5 h and dissolved in 5 ml of 0.3025 M HCl (Rehchigl and Payne 1990) and total P concentrations determined using the molybdenum blue method (Murphy and Riley 1962). The agronomic P use efficiency (APE) is a measure of the increase in yield per unit of added P fertilizer (g DM g<sup>-1</sup> P) (Akhtar et al. 2009). The APE was calculated as follows: APE (g DM g<sup>-1</sup> P) = [DM (root + shoot) on fertilized soil - DM (root + shoot) on unfertilized soil] / amount of P applied.

Soil P fraction via sequential chemical extraction

Soil P fractions were determined following a modified Hedley fractionation method (Hedley et al. 1982) which segregates P into 6 operationally defined fractions. Prior to extraction, the collected soils were air-dried, and mixed thoroughly. Half a gram of ground soil (<0.148 mm) was suspended in 30 ml deionized water along with 2 HCO<sub>3</sub><sup>-</sup>-saturated resin strips (anion-exchange membrane, 9×62 mm) and shaken on a rotary flat-bed shaker for 16 h. Resin strips were removed and placed in 30 ml of 0.5 mol l<sup>-1</sup> HCl to desorb P; this P fraction is referred to as resin P. The soil suspension remaining was centrifuged and the supernatant discarded. The soil pellet was then successively extracted with 30 ml of 0.5 M NaHCO<sub>3</sub> (pH 8.5), followed by 30 ml of 0.1 M NaOH, and 30 ml of 1 M HCl each for 16 h. The supernatants from each of these steps were collected by centrifugation and the inorganic P (P<sub>i</sub>) concentration determined using the molybdenum blue method. These fractions are referred to as NaHCO<sub>3</sub>-P<sub>i</sub>, NaOH-P<sub>i</sub> and HCl-P<sub>i</sub> fractions. Total P concentration of NaHCO<sub>3</sub> and NaOH extracts were determined after acid ammonium persulfate digestion (USEPA 1971). Organic P (P<sub>o</sub>) concentration of these extracts was calculated as the difference between the total and inorganic P content. They are referred to as NaHCO<sub>3</sub>-P<sub>o</sub> and NaOH-P<sub>o</sub> fractions, respectively. The sum of resin-P, NaHCO<sub>3</sub>-P<sub>o</sub> and NaHCO<sub>3</sub>-P<sub>i</sub> was defined as plant available P (Cross and Schlesinger 1995).

### Potential acid phosphatase (AcPase) assays

AcPase was assayed following the fluorimetric enzyme assay method of Saiya-Cork et al. (2002) with minor modification. Briefly, soil sample suspensions were prepared by mixing 1 g soil with 125 mM (pH 6.0) sodium acetate buffer in a Nalgene bottle and homogenizing for two and half minutes (800 rpm) on a magnetic stir plate. The resulting suspensions were transferred to a glass crystalizing dish and continuously stirred at 100 rpm while 200  $\mu$ l aliquots were dispensed into a black 96-well microplate. Blank wells only received 250  $\mu$ l buffer while reference standard wells received 50  $\mu$ l MUB standard (100  $\mu$ M 4-methylumbelliferone) plus 200  $\mu$ l buffer. Negative control wells received 50  $\mu$ l substrate (200  $\mu$ M 4-methylumbelliferyl phosphate) plus 200  $\mu$ l buffer. Quench wells contained 50  $\mu$ l MUB standard plus 200  $\mu$ l soil suspension while positive control wells received 50  $\mu$ l buffer and 200  $\mu$ l soil suspension. Assay wells received 50  $\mu$ l substrate plus 200  $\mu$ l of the soil suspensions. After the microplate was prepared, it was incubated in the dark at 20 °C for 30 min. After 10 min a 10  $\mu$ l aliquot of 0.5 M NaOH was added to each well to stop the reaction and maximize MUB fluorescence. Fluorescence was then measured immediately using Perkin-Elmer Wallac Victor2 1420 multi-label counter with 355 nm excitation and 450 nm emission filters. AcPase was expressed in units of  $\text{nmol h}^{-1} \text{g}^{-1}$ .

### Rhizosphere microbial community composition - PLFA analysis

Specific fatty acid methyl esters (FAMES) in the soils were identified and quantified using the high throughput protocol described by Buyer and Sasser (2012). Briefly, FAMESs were extracted from the soil with 4.0 ml of 50 mM Bligh-Dyer (1:2:0.8 (v/v/v), chloroform/methanol/phosphate buffer; pH 7.4) extractant spiked with 19:0 (1,2-dinonadecanoyl-sn-glycero-3-phosphocholine) internal standard by first sonicating the mixture for 10 min followed by rotating end-over-end for 2 h. Samples were centrifuged and the liquid phase transferred to a 13  $\times$  100 mm test tubes with PTFE lined screw cap to which 1 ml chloroform and water were added. The lower phases containing the FAMES was collected, dried in a CentriVap (Labconco) and the dry powder dissolved in 1 ml chloroform. Lipids were separated by loading the samples onto a preconditioned 96-well solid phase extraction (SPE) plate

(Phenomenex, Torrance, CA, USA) followed by washing with 1 ml of chloroform and 1 ml acetone and then elution of the FAMES by 0.5 ml of methanol:chloroform:H<sub>2</sub>O (5:5:1). Finally, 0.2 ml of transesterification reagent was added and the samples incubated at 37 °C for 15 min. After incubation 0.4 ml of 0.075 M acetic acid and 0.4 ml chloroform were added and the bottom phase removed and dried. The samples were redissolved in 75  $\mu$ l of hexane, transferred to glass inserts in GC vials, and the FAMES identified using a MIDI system (Microbial Identification System Inc., Newark, DE) consisting of an Agilent 7890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with a 100 place autosampler, an Agilent 7693 Ultra 2 column, and flame ionization detector. The carrier gas was ultra-high-purity hydrogen gas with a column split ratio of 30:1 and a flow rate of 1.2  $\text{ml min}^{-1}$ . The oven temperature was raised from 190 to 285 °C at 10 °C  $\text{min}^{-1}$  and then to 310 °C at 60 °C  $\text{min}^{-1}$  where it was held for 2 min. The injector and detector temperatures were 285 and 300 °C, respectively. FAME identities and relative percentages were automatically calculated using MIDI methods (Sherlock Microbial Identification System version 6.2, MIDI Inc., Newark, DE) described by Buyer and Sasser (2012).

The individual FAMES a12:0, i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, i18:0, i15:1 $\omega$ 6c and i17:1 $\omega$ 9c represent Gram positive (G+) bacteria; 16:1 $\omega$ 9c, 16:1 $\omega$ 7c, 17:1 $\omega$ 8c 18:1 $\omega$ 9c, 18:1 $\omega$ 5c, 20:1 $\omega$ 9c, cy17:0 $\omega$ 7c, cy19:0 $\omega$ 9c, cy19:0 $\omega$ 7c represent Gram-negative bacteria (G-); 10me16:0, 10me17:1 $\omega$ 7c, 10me17:0, 10me18:1 $\omega$ 7c, 10me18:0, 10me19:1 $\omega$ 7c represent actinobacteria; 18:3 $\omega$ 6c, 19:3 $\omega$ 3c, 20:5 $\omega$ 3c, 20:2 $\omega$ 6c represent eukaryotes. The FAME 16:1 $\omega$ 5c is used as a measure of arbuscular mycorrhizal fungi (AM fungi) while the polyenoic, unsaturated FAME 18:2 $\omega$ 6,9c was used to represent general fungi because it is suggested to be mainly of fungal origin in soil (Myers et al. 2001; Ding et al. 2011). Total microbial biomass (TMB) was calculated by summing the concentration of the identified 47 fatty acids, and was expressed as  $\text{nmol PLFAs g}^{-1} \text{soil}$ .

### Statistics

Prior to analysis of variance (ANOVA), data were checked for normal distribution and log transformed where necessary. ANOVA was performed with JMP statistical software (version 10.0, SAS Institute 2012)

to test for the fixed effects of P source, endophyte strain, and their interaction on tall fescue biomass, P removed and concentration in root and shoot, soil P fractions, AcPase and PLFA concentrations. Linear contrasts were used to determine changes in soil P fractions, pH and PLFA concentrations between rhizosphere and pre-planted soils. Stepwise regression procedures were used to investigate the relationship of individual fatty acids with P acquisition and plant biomass production, respectively (SPSS, version 16.0).

Univariate analyses testing for treatment effects on microbial biomarker groups were conducted using a generalized linear mixed model (PROC GLIMMIX, SAS v.9.3), which does not require that the original data be normally distributed. Lognormal and beta distributions with identity and logit link functions, respectively, were fitted to the microbial biomarker group concentration and proportion data, respectively. Where significant, differences between the means were determined using the student's *t*-test at a probability level of  $p < 0.05$ .

To test for community level differences between treatments, PLFA concentrations were first Hellinger transformed (Ramette 2007) before creating a non-metric multidimensional scaling (NMDS) ordination with Sorensen (Bray-Curtis) distances using the slow and thorough settings of the autopilot mode in PC-ORD (v.6.08). A multi-response permutation procedure (MRPP) was used to determine the difference between data groups. A Bonferroni's correction was applied to the *p*-values to correct for multiple comparisons. In MRPP, a small *p*-value indicates that the predefined grouping variables (soil region, cultivar, endophyte or cultivar, and endophyte pairs) are more different than expected by chance. The effect size is reflected in the 'A'-value, the chance corrected within-group agreement, which indicates the similarity of samples within a group. An 'A'=1 if the samples in a group are identical, and 'A' is closer to zero if their heterogeneity is higher than expected by chance. An 'A' value  $> 0.3$  for ecological data is considered high.

Relationships between environmental variables and PLFA biomarker groups were determined by correlating soil and plant variables (P fractions, pH, AcPase, tall fescue biomass, and P uptake) with axis scores in the NMS ordination. Those parameters with an associated  $r^2$  value of 0.400 or greater were overlaid on the NMS ordination as a biplot and displayed as vectors. The direction and length of the vectors indicates the

relationship (positive or negative) and strength of the correlation, respectively, while the angle between vectors indicates the correlation between the variables.

## Results

### Plant growth, P acquisition and APE

P source significantly influenced tall fescue root and shoot dry biomass and P uptake (Table 1). Tall fescue biomass (root and shoot) was significantly greater in K-Ps, Al-Ps, and Ca-Ps treatments compared with those grown in control and Fe-Ps soils, respectively (Fig. 1a), in which plants showed visual symptoms of P deficiency including yellow foliage and reduced tillering (Fig. 2). Endophyte infection significantly influenced only shoot biomass (Table 1). Tall fescue grown in K-Ps, Al-Ps and Ca-Ps treatments removed more P per pot compared to those cultivated in Fe-Ps and control soils (Fig. 1b). AR542E+ infected tall fescue produced significantly less shoot biomass than E- and CTE+ infected tall fescue, but similar amounts to that of AR584E+ infected tall fescue (Fig. 3). There was a significant interaction between P source and endophyte status on the concentration of P in the root tissues (Table 1). Notably, infection with novel endophyte AR542E+ resulted in significantly lower root P concentrations when grown in the Fe-Ps treatment (Fig. S1).

P form was also a key factor influencing APE (Table 1). APE in Fe-Ps treatment was only 10.42 g DM P g<sup>-1</sup>, which was significantly lower (~15 fold) than those in soils receiving Ca-Ps, K-Ps, and Al-Ps forms (Fig. 4). Endophyte and the endophyte x P form interaction had no effect on total tall fescue biomass, P acquisition, or APE.

### Soil P fractions

The total P extracted from the pre-planted soils receiving the K-Ps treatment was 100 %, which was significantly higher than those soils receiving the Al-Ps (81 %), Fe-Ps treatments (81 %) and Ca-P (37 %) treatments (Table 2). The available P concentrations (sum of resin P, NaHCO<sub>3</sub>-P<sub>i</sub>, and NaHCO<sub>3</sub>-P<sub>o</sub>) in soils followed the order: K-Ps > Ca-Ps > Al-Ps > Fe-Ps = control (Table 2). Of the three P fractions making up available P in the pre-planted soils, the organic P (NaHCO<sub>3</sub>-P<sub>o</sub>) fraction was greatest; but it was not significantly

**Table 1** Analysis of variance summary testing the main effects of P source (P; K-Ps, Fe-Ps, Al-Ps, Ca-Ps), endophyte infection status (E; E-, CTE+, AR542E+, AR584E+) and their interaction on plant biomass, P removal and concentration in roots and shoots, agronomic P

Effect	Dry Mass			P uptake		APE	P fractions						
	Root	Shoot	Total	root	shoot		Resin P						
						NaHCO <sub>3</sub> -P <sub>i</sub>	NaHCO <sub>3</sub> -P <sub>o</sub>	Available P	NaOH-P <sub>i</sub>	NaOH-P <sub>o</sub>	HCl-P		
					μg pot <sup>-1</sup>	μg g <sup>-1</sup>							
P source (P)	**	**	NS	**	**	**	**	**	**	**	**	NS	NS
Endophyte (E)	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
P x E	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

\*\* indicates significant difference at  $p < 0.01$ , \* indicates significant difference at  $p < 0.05$

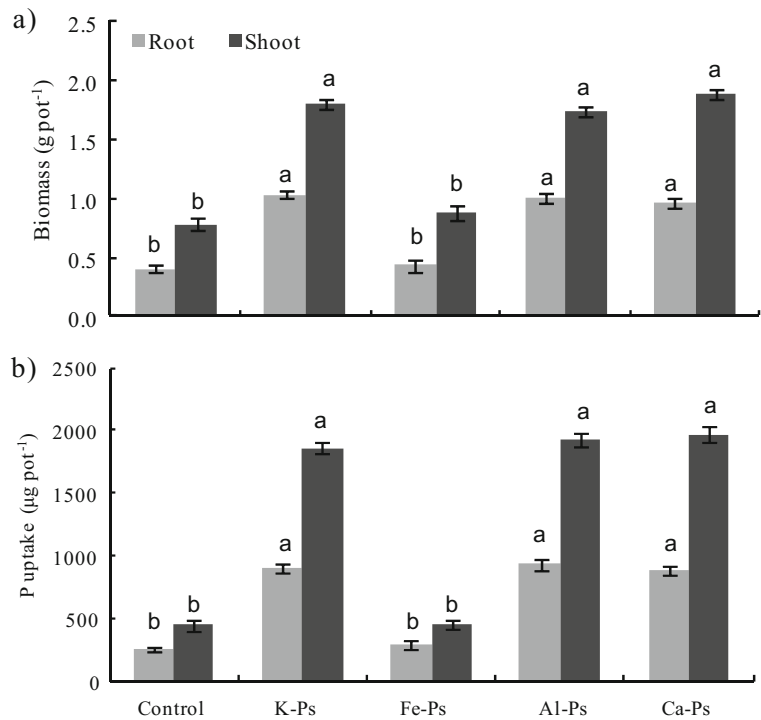
different between the P treatments. In the remaining available P fractions (resin P, NaHCO<sub>3</sub>-P<sub>i</sub>), more of the Al-Ps was in the NaHCO<sub>3</sub>-P<sub>i</sub> fraction compared to Ca-Ps, in which the greatest proportion was found in the most readily available resin P fraction. Adding Fe-Ps resulted in a significant increase in the NaOH-P<sub>i</sub> fraction compared with the control soils and other P forms. There was no significant difference in the organic P (NaOH-P<sub>o</sub> and NaHCO<sub>3</sub>-P<sub>o</sub>) or the HCl-P fractions in any P treatment.

Tall fescue growth for 90 days in all treatments but K-Ps resulted in a significant pH reduction (Table 2). The P extraction percentages in Fe-Ps and Ca-Ps soils were 101 and 85 %, respectively, which were markedly higher than those in pre-planted soils. Significant reduction in the resin P fractions and an increase in the organic P fractions (NaHCO<sub>3</sub>-P<sub>o</sub>, NaOH-P<sub>o</sub>) were observed in all P treatments. The non-labile HCl-P fraction was not influenced by the plant, regardless of the P source. Endophyte infection and the endophyte x P form interaction had no effect on the P fractions after 90-days of growth, with the exception of NaOH-P<sub>i</sub> fraction (Table 1). The greatest NaOH-P<sub>i</sub> fraction was found in the rhizosphere of E- plants, which was similar to the rhizosphere of CTE+ and AR584E+ but significantly higher than in rhizosphere soils of AR542E+ infected plants (Fig. 5). Further, linear contrast analysis comparing soils with endophyte-infected (CTE+, AR542E+, and AR584E+) rather than endophyte free (E-) tall fescue showed that endophyte infection significantly reduced the NaOH-P<sub>i</sub> fraction.

#### Potential acid phosphatase activity (AcPase)

AcPase in the rhizosphere was strongly affected by the interaction of P forms and endophyte strain (Table 3 and Fig. 6). AcPase was greatest in control soils, regardless of endophyte strains, while the K-Ps treatment had the lowest values. In Fe-Ps treatment, AcPase in the rhizosphere of AR542E+ infected tall fescue (222.5 nmol h<sup>-1</sup> g<sup>-1</sup>) was significantly lower than in soils of E- (448.9 nmol h<sup>-1</sup> g<sup>-1</sup>), CTE+ (416.8 nmol h<sup>-1</sup> g<sup>-1</sup>) and AR584E+ (414.5 nmol h<sup>-1</sup> g<sup>-1</sup>) infected tall fescue. The same trend was also observed in the Ca-Ps treatment. Alternatively, AcPase in the rhizosphere of AR584E+ in the Al-Ps and K-Ps treatments was lower compared to the other endophyte strains (CTE+ and AR542E+) and E-.

**Fig. 1** The influence of P source on **a** root and shoot biomass production and **b** total amount of P removed in root and shoot per pot. Means in the same plant part with different letters are significant different ( $p < 0.05$ ). Bar represents  $\pm$  the standard error of the mean



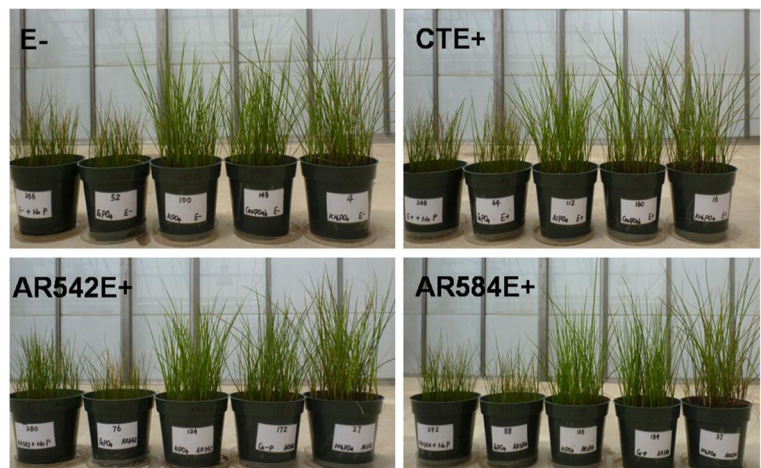
### Microbial community composition

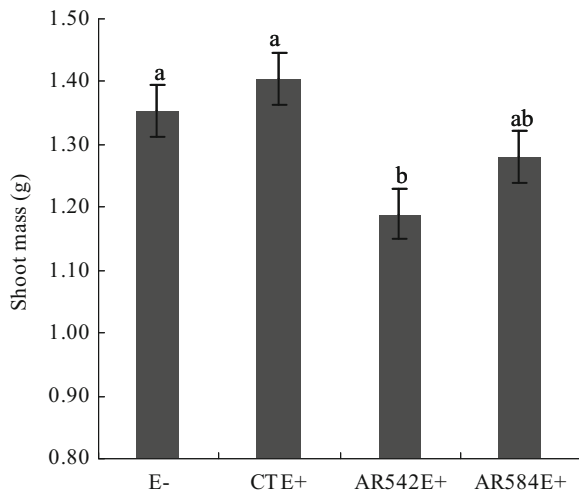
After 90-days of tall fescue growth, soil microbial composition was determined using PLFA analysis. P form strongly influenced TMB, G<sup>+</sup>, G<sup>-</sup>, and AM fungi concentrations in rhizosphere soils while the effects of endophyte infection and the endophyte  $\times$  P form interactions were not significant (Table 3). G<sup>+</sup>, G<sup>-</sup>, actinobacteria, general fungi, and TMB in rhizosphere soils receiving the K-Ps, Al-Ps and Ca-Ps treatments

were significantly greater than those in Fe-Ps and control soils (Table S1). AM fungi concentrations were highest under the K-Ps treatment followed by Ca-Ps and Al-Ps, all of which were significantly greater than the Fe-Ps and control treatments.

Linear contrasts between the pre-planted and rhizosphere soils showed how the microbial community composition changed due to 90 days of tall fescue growth in the different P treatments. In the control treatment, only the G<sup>+</sup> biomarker group significantly increased while all

**Fig. 2** Comparison of tall fescue growth with four P sources (left to right: control, Fe-Ps, Al-Ps, Ca-Ps and K-Ps)

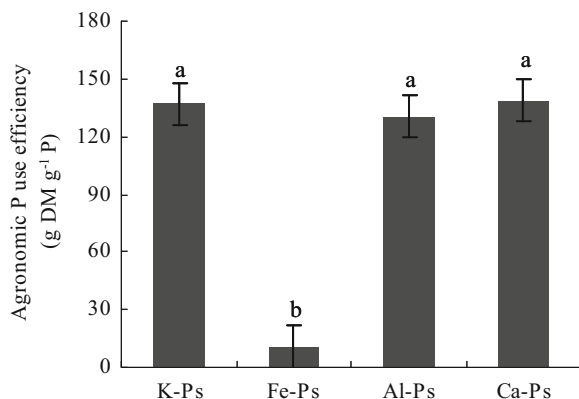




**Fig. 3** Influence of fungal endophyte infection (E-, CTE+, AR542E+ and AR584E+) on shoot biomass of tall fescue per pot. Means followed by different letters are significantly different ( $p < 0.05$ ). Bar represents  $\pm$  the standard error of the mean

other microbial parameters remained the same (Table S1). In all treatments receiving additional P, the G+ biomarker group and TMB significantly increased (Table S1). The K-Ps treatment had the greatest influence on soil microbial community, in which all microbial parameters significantly increased. Changes in microbial biomarker group concentrations were less consistent for the other P treatments (Table S1).

The NMDS plots (Fig. 7) and MRPP analysis based on all P treatments and control soils show that the microbial community structure evidenced by PLFA analysis in the soils receiving K-Ps, Al-Ps and Ca-Ps were similar ( $'A' = -0.02$ – $0.11$ ;



**Fig. 4** The influence of P source (K-Ps, Fe-Ps, Al-Ps, or Ca-Ps) on mean agronomic P efficiency (APE) (g DM g<sup>-1</sup> P) of tall fescue. Means with different letters are significantly different ( $p < 0.05$ ). Bar represents  $\pm$  the standard error of the mean

$p > 0.05$ ), but significantly different ( $'A' = 0.12$ – $0.24$ ;  $p < 0.01$ ) from the microbial community in control and those soils receiving Fe-Ps which were highly similarity ( $'A' = 0.0052$ ;  $p = 0.56$ ). Biplots using the P fractions and tall fescue growth parameters show that root mass ( $r = 0.919$ ), shoot mass ( $r = 0.907$ ), P in shoot ( $r = 0.918$ ) and P in root ( $r = 0.928$ ) are positively correlated with axis 1, which explained 87.2 % of the variation (Fig. 7a) corresponding to the addition of K-Ps, Al-Ps, and Ca-Ps forms. Conversely, AcPase was negatively correlated ( $r = -0.654$ ) with axis 1 and in the direction of the control soils and those receiving Fe-Ps additions. Similarly, biplots using the concentration of PLFA biomarker groups show that total microbial biomass, G+, G-, AM fungi, general fungi, actinobacteria, and eukaryotes are positively correlated with axis 1 and in the direction of the K-Ps, Al-Ps and Ca-Ps additions (Fig. 7b). The NMDS plots and MRPP analysis within P treatment (Fig. S2) further highlight that PLFA profiles in soils receiving K-Ps, Al-Ps and Ca-Ps additions are significantly different from soils with no P addition. However, such effect was not seen in soils receiving Fe-Ps addition.

Stepwise regressions were used to identify if any individual microbial fatty acids were correlated to tall fescue growth and P uptake when grown with different P sources (Table 4). Interestingly, of all identified fatty acids, the fatty acid 16:1 $\omega$ 5c, a typical biomarker for AM fungi, correlated best with P acquisition and plant biomass production. The correlation coefficient for both the multivariate correlation and partial regression were significant ( $p < 0.05$ ).

## Discussion

Tall fescue biomass production, P acquisition, and changes in P fractions

Fungal endophyte infection in tall fescue is thought to improve the host plant's ability to access P from soils by altering root system architecture, or by the release of phenolic-like compounds capable of reducing or chelating metals (e.g., Fe and Al) binding soil P (Malinowski et al. 1998a, 1999). We showed in previous studies with the same cultivar-endophyte combinations used in this study that root exudate chemistry varied with endophyte

**Table 2** P fractions (mg kg<sup>-1</sup> soil) and pH in pre-planted soils (14 days after P addition before planting), and rhizosphere soils after 90-days of tall fescue. Summary of *p* values generated by linear contrast analysis determining the effects of tall fescue

growth on P fractions by comparing P fractions in rhizosphere soils after 90-days of tall fescue growth to pre-planted soils. Numbers in parentheses indicate the % change between pre-planted and rhizosphere soils

P form	pH	Resin-P	NaHCO <sub>3</sub> -P <sub>i</sub>	NaHCO <sub>3</sub> -P <sub>o</sub>	Available P	NaOH-P <sub>i</sub>	NaOH-P <sub>o</sub>	HCl-P	Total P
Pre-planted soil (mg · kg <sup>-1</sup> soil)									
Control	5.6a	6.5d	5.1c	23.6a	35.4c	46.4c	153.8a	10.6a	246.3c
K-Ps	5.6a	21.0a	9.3a	25.1a	56.0a	58.8b	151.a	10.3a	276.2a (100) <sup>a</sup>
Fe-Ps	5.6a	6.5d	5.0c	23.6a	35.1c	70.3a	153.7a	11.6a	270.7a (81)
Al-Ps	5.7a	12.0c	10.7a	24.4a	47.0b	59.2b	150.9a	13.52a	270.6a (81)
Ca-Ps	5.8a	17.6b	7.4b	25.1a	50.1b	46.4c	147.5a	13.43a	257.5b (37)
Rhizosphere soil (mg · kg <sup>-1</sup> soil)									
Control	5.2c	3.4c	5.8c	25.8c	35.1c	47.9c	160.9a	11.4a	255.3b
K-Ps	5.7a	7.1a	9.3a	30.9a	47.1a	54.2b	165.7a	12.0a	279.0a (79)
Fe-Ps	5.5b	3.9c	7.4b	28.bc	39.3b	69.3a	163.2a	13.7a	285.5a (101)
Al-Ps	5.5b	5.9b	8.7a	30.7a	45.3a	56.3b	164.3a	12.5a	278.5a (77)
Ca-Ps	5.6ab	5.9b	8.7a	29.6ab	44.2a	57.2b	164.6a	14.9a	280.8a (85)
Rhizosphere soil vs. Pre-planted soil									
Control	** (-7)	** (-47)	NS	* (+9)	NS	NS	NS	NS	NS
K-Ps	NS	** (-65)	NS	* (+18)	** (-16)	NS	** (+9)	NS	NS
Fe-Ps	* (-2)	* (-40)	** (+33)	** (+16)	* (+11)	NS	* (+6)	NS	** (+5)
Al-Ps	** (-3)	** (-51)	* (-18)	** (+21)	NS	NS	** (+8)	NS	NS
Ca-Ps	* (-2)	** (-67)	* (+15)	** (+15)	** (-12)	** (+19)	** (+10)	NS	** (+8)

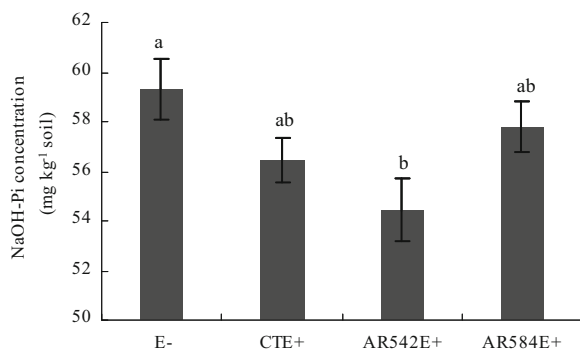
\*\* indicates significant different at  $p < 0.01$ , \* indicates significant different at  $p < 0.05$ , Means followed by different letters within column are significantly different ( $p < 0.05$ )

<sup>a</sup> P extraction percentage (%) = (total P in P treatment - total P in control) / total P added (30 mg kg<sup>-1</sup>) × 100 %

genotype (Guo et al. 2015) as did root system architecture in response to different P sources (Ding et al. 2015). Based on these previous findings, we hypothesized that endophyte infection would enhance P uptake and plant

biomass production in a P form and endophyte genotype dependent manner.

In contrast to our prediction, endophyte genotype and P forms did not interact to influence plant biomass production. Instead, CTE+ infected plants tended to produce more shoot biomass while the novel endophyte infected plants tended to produce less relative to E-plants irrespective of P sources. Infection with novel endophyte AR542E+ resulted in significantly less shoot biomass production compared to E- and CTE+ infected plants. Our finding is consistent with several studies (Belesky and Burner 2004; Rudgers et al. 2010; Guo et al. 2015), which reported that AR542E+ infected tall fescue produced less biomass than other endophyte infected strains. Such interesting findings suggest that hosting this particular novel strain might impose a metabolic cost to the plant (Belesky and Burner 2004). The reduction in shoot biomass for this combination may explain some of the differences we observed in P uptake and soil P fractions discussed below.



**Fig. 5** Influence of fungal endophyte infection (E-, CTE+, AR542E+ and AR584E+) on the soil NaOH-P<sub>i</sub> fraction. Means with different letters are significantly different ( $p < 0.05$ ). Bar represents ± the standard error of the mean

**Table 3** Analysis of variance summary testing the fixed effects of P source (P), endophyte (E) and their interactions on concentrations of PLFA biomarkers groups (G+, G-, eukaryote, AM fungi,

actinobacteria, fungi), total microbial biomass (TMB) and potential acid phosphatase activity (AcPase) in rhizosphere soils from tall fescue after 90 days of growth

Effect	G+	G-	Actinobacteria	AM fungi	Fungi	Eukaryote	TMB	AcPase
P source (P)	**	**	NS	**	NS	NS	**	**
Endophyte (E)	NS	NS	NS	NS	NS	NS	NS	**
P x E	NS	NS	NS	NS	NS	NS	NS	**

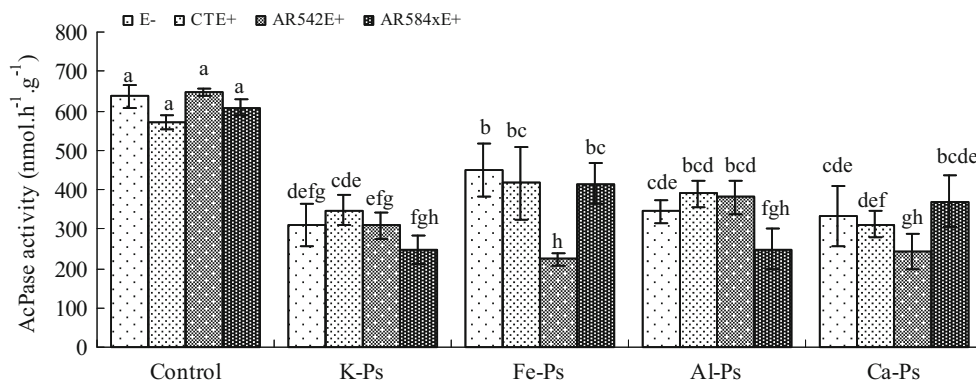
\*\* indicates significant difference at  $p < 0.01$ , \* indicates significant difference at  $p < 0.05$ 

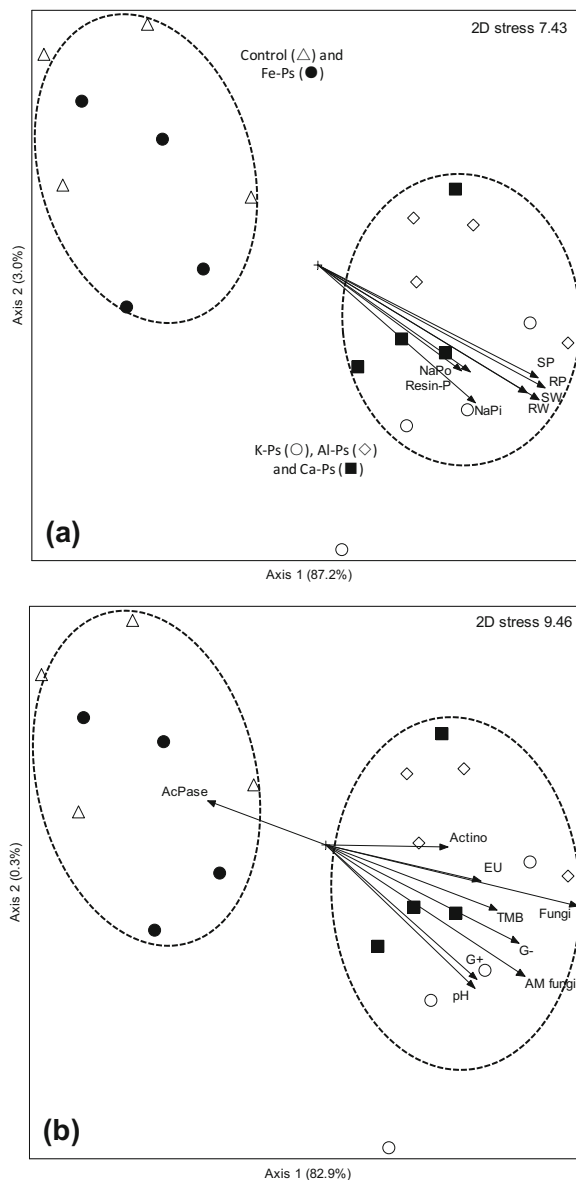
The concentration of P in the roots was significantly, but inconsistently, influenced by the interaction of endophyte genotype and P source. All plants grew poorly in the Fe-Ps treatment, especially the AR542E+ infected plants, which (while having the same root mass as the others) had the lowest P concentration. While the novel AR542E+ endophyte may provide significant advantages over CTE+ infected tall fescue by not producing toxic ergot alkaloids, the building evidence suggest that the PDF/AR542E+ combination cannot be expected to produce the same amount of shoot biomass as the novel AR584E+ infected combination.

Overwhelmingly, P forms had the greatest influence on all parameters measured. We predicted that the less soluble P forms (e.g., Ca-Ps, Al-Ps and Fe-Ps) would be less bioavailable compared to K-Ps, resulting in (even with any advantage from the endophyte) less P uptake and plant biomass production. We found that the overall extraction efficiencies for the Ca-Ps, Al-Ps and Fe-Ps treatments in the pre-planted soils were considerably less than the K-Ps treatment; especially for Ca-Ps where only 37 % of the total P added was recovered. The overall extraction efficiencies mimic the predicted

solubility of the different P compounds used and, based on this result, we might have expected that plant growth would follow a similar trend (i.e., less plant growth and P uptake in soils with the least P extracted). However, in the treatments with Ca-Ps and Al-Ps we found that the plants grew just as well as those grown with readily available K-Ps. There was no significant endophyte effect to explain this response, but instead, the explanation was found in the P fractions data.

Al-Ps and Ca-Ps are commonly assigned to stable P pools that are considered sparing available to plants (Wang et al. 2011). Interestingly, in current study, a significant proportion of the total P in the pre-planted soils in Al-Ps and Ca-Ps treatments was recovered in the available P fraction (summation of resin P,  $\text{NaHCO}_3\text{-P}_i$  and  $\text{NaHCO}_3\text{-P}_o$ ) indicating that sufficient dissolution of these P forms occurred prior to planting likely due, in part, to the acidic pH of the soils and/or the crystallinity of the source material, or enhanced mineralization in the initially disturbed soils. The higher than expected plant-available resin-P concentrations likely contributed to P uptake and plant growth in the Al-Ps and Ca-Ps treatments similar to those receiving K-Ps. This finding is

**Fig. 6** Potential acid phosphatase activity (AcPase,  $\text{nmol h}^{-1} \text{g}^{-1}$ ) in the rhizosphere of tall fescue (E-, CTE+, AR542E+ and AR584E+) under P sources (K-Ps, Fe-Ps, Al-Ps and Ca-Ps) andcontrol soils. Means with different letters are significantly different ( $p < 0.05$ ). Bar represents  $\pm$  the standard error of the mean



**Fig. 7** Nonmetric multidimensional scaling (NMS) biplots using PLFA biomarker group relative abundance in soils sampled from control (No-P) treatment and those receiving different P sources (Fe-Ps, K-Ps, Al-Ps and Ca-Ps). Panel **a** includes correlation vectors for P fractions, shoot weight and shoot P concentration, and panel **b** includes correlation vectors for PLFA biomarker groups, pH and AcPase. Symbols represent the mean of four replicates. Radiating lines from the ordination centroid indicate the strength and direction of Pearson correlations ( $r^2 > 0.4$ ) between variables and axis scores. *SP* P in shoot, *RP* P in root, *SW* shoot weight, *RW* root weight, *TMB* total microbial biomass, *Actino* actinobacteria, *EU* eukaryote, *NaPi*  $\text{NaHCO}_3\text{-P}_i$ , *NaPo*  $\text{NaHCO}_3\text{-P}_o$

consistent with our previous in-vitro study using the same tall fescue cultivar-endophyte combinations

grown in agar with the same P treatments and pH (~5.5) (Ding et al. 2015).

Most of the P accessed by the plants appears to have come from the most available soil P fractions. However, the specific available fractions used by the plants depended on P forms. For example, in the K-Ps treatment considerably more P in the pre-planted soil was in the most available, resin-P fraction, which was significantly depleted (~65 %) after 90 days of plant growth, while no significant reduction was found in the other available P fractions for this treatment. However, in the pre-planted soils receiving the Al-Ps treatment there was significantly less P in the resin-P fraction relative to the K-Ps treatment, in which case plant growth in the soils receiving Al-Ps appears to have resulted in depletion (~18 %) of P from the  $\text{NaHCO}_3\text{-P}_i$  fraction. For the Ca-Ps treatment, the P concentration in the resin P fraction of the pre-planted soils was second only to the K-Ps treatment and was reduced the most (–67 %) after plant growth. Plant growth in the Ca-Ps treatment also resulted in an overall increase in the total amount of P recovered (85 vs. 37 % in the pre-planted soils). The greater P recovery after plant growth is likely due to an increase in the amount of P released from the residual Ca-Ps fraction due to rhizosphere processes (i.e., root exudates, AMF colonization, etc.) after which the liberated P was then re-partitioned into the other P fractions (notably  $\text{NaOH-P}_i$ ).

Another expectation from this study was that endophyte infection would significantly influence tall fescue's ability to access P. We found, irrespective of the type of P added to the soil, that endophyte infection resulted in an overall reduction of P from the  $\text{NaOH-P}_i$  fraction relative to E- plants; however only infection with AR542E+ was this decrease (~8 %) significant. Endophyte-driven decrease in the  $\text{NaOH-P}_i$  fraction suggests that endophyte infection in general, and AR542E+ in particular, may in some way lead to repartitioning of P to other P fractions. To truly track the redistribution would require testing using, for example,  $^{32}\text{P}$  or  $^{33}\text{P}$  labeled fertilizers. There were significant increases in the  $\text{NaHCO}_3\text{-P}_o$  and  $\text{NaOH-P}_o$  fractions which would imply that P was being retained in either root or microbial biomass. There was no consistent endophyte effect on root biomass, P concentration, or total P that correlated with increased P in the organic fractions, which might indicate repartitioning of P from the  $\text{NaOH-P}_i$  fraction to soil microbes (i.e., immobilization).

**Table 4** Stepwise regression analysis for predicting plant growth (root and shoot biomass) and P uptake (P removed in roots and shoots) in tall fescue based on individual phospholipid fatty acids (PLFAs)

Stepwise regression models <sup>a</sup>	F value	T value and r of the partial regression coefficient	
		T value	r
$Y_1 = 348.04X_1 - 949.87$	106.576**	10.324	0.760
$Y_2 = 762.96X_1 - 2171.31$	104.329**	10.214	0.756
$Y_3 = 0.34X_1 - 0.77$	108.391**	10.411	0.763
$Y_4 = 0.56X_1 - 1.131$	134.280**	11.588	0.795

\*\* correlation is significant ( $p < 0.01$ ). <sup>a</sup>  $Y_1$  P content in root ( $\mu\text{g}$  per pot),  $Y_2$  P content in shoot ( $\mu\text{g}$  per pot),  $Y_3$  root weight (g per pot),  $Y_4$  shoot weight (g per pot),  $X_1$  16:1 $\omega$ 5c (nmol  $\text{g}^{-1}$ )

The NaOH- $\text{P}_i$  fraction represents P associated with Fe and Al at mineral surfaces (Maher and Thorrold 1989) and is usually considered to be part of the non-labile P fraction (Hassan et al. 2012). Previous studies implicated phenolic-like compounds with iron (Fe) reducing and aluminum (Al) and copper (Cu) chelating activities, only found in the root exudates of endophyte infected tall fescue, for their role in enhancing P acquisition (Malinowski et al. 1998a). In a recent study using PDF with the same endophytes we showed that endophyte genotype influenced the composition of root exudates; some of which are known to play a role in P acquisition (Guo et al. 2015). Our findings support endophyte-enhanced removal of P (particularly by AR542E+) from this unavailable soil P fraction. However, in the timeframe and system we were studying, it did not translate to greater P uptake or shoot biomass in AR542E+ infected tall fescue. Instead, the PDF/AR542E+ combination produced the least biomass. One possibility in this case is that the multifaceted interaction (physiological and metabolic) between the PDF/AR542E+ pair results in reduced shoot biomass and changes in root architecture and exudate composition which in turn uniquely alters soil P fractions.

Unlike plants grown in the Al-Ps and Ca-Ps treatments, plants grown with Fe-Ps took up significantly less P, had the least biomass production, and lowest APE, together with typical symptoms of P deficiency, regardless of endophyte infection. These findings clearly indicate that plants, even with endophyte infection, were not able to access P when present as iron phosphate which highlights the common problem of P-fixation in acidic soils (Ghosh et al. 1996).

#### Linking soil P fractions, P uptake and plant growth to rhizosphere processes

We expected that soil microbial community structure and function would vary in an endophyte strain and P form dependent manner resulting in differences in P uptake and plant growth. However, the type of P added to the soils had an overwhelming effect on all parameters tested while effects of endophyte infection were minor.

Plants have developed various mechanisms to enhance the acquisition of P from soils. Phosphatase production is considered to be one component of a plant phosphate-starvation rescue system (Wasaki et al. 2003). Overall potential AcPase activity was significantly greater when P availability was low (i.e., in control and Fe-Ps treated soils) which has been found in many studies involving a variety of plants (Tadano and Sakai 1991; Tadano et al. 1993; Sinsabaugh 1994). While P availability in this study was clearly a factor influencing potential AcPase activity, the inconsistent relationships we found between P forms and endophyte status could likely be attributed to several other environmental factors (Li et al. 2012).

Besides AcPase production in the rhizosphere, soil microorganisms also play a critical role in maintaining soil function (Garbeva et al. 2004), because they are involved in mineralizing nutrients required for plant growth (Song et al. 2007). However, understanding the relationships between the composition of the microbial community in the rhizosphere of endophyte infected tall fescue plants and the endophyte's role in assisting tall fescue with nutrient acquisition (P mobilization) remains poorly understood; particularly in the rhizosphere of novel endophyte-infected tall fescue.

Several studies have suggested that in response to P deficiency endophyte infection results in enhanced production of phenolics and other root exudates, such as organic acid anions or carboxylates, which could influence the structure and function of the soil microbial community (Van Hecke et al. 2005). However, in our study, we failed to detect any influence of endophyte infection on microbial community composition. The quantity of rhizodeposits always coincides with plant root growth, and composition with plant developmental stage both of which strongly influences the diverse and dense microbial community (Grayston et al. 1998; Treonis et al. 2005). Reasons for not seeing the expected differences in microbial community structure could then have to do with us only sampling at one time point (90 days), and there being no differences in root biomass among the endophyte-cultivar pairs.

In this study, soil microbial communities were strongly influenced by P forms. Previous studies reported that P form significantly changed carboxylate exudation by growing plants (Li et al. 2010), which might exert a selection on root-associated microorganisms. The four forms of P used in the present experiment differed in availability, which altered plant growth, and hence, may partly explain the differential effects on microbial community compositions and abundance. Rhizosphere soil microorganisms might be involved in P mobilization for tall fescue growth. For example, stepwise regression analysis showed that the lipid 16:1 $\omega$ 5c, a typical biomarker of AM fungi, was effective at predicting shoot biomass, and P concentrations in tall fescue tissues. An abundance of physiological and molecular research has clearly demonstrated that AM fungi contribute significantly to the P nutrition of plants, particularly under low P conditions (Tawaraya et al. 2006; Richardson et al. 2009, 2011; He et al. 2013). Most studies to date have shown a negative influence of endophyte infection on AMF colonization (Buyer et al. 2011; Chu-Chou et al. 1992; Guo et al. 1992; Mack and Rudgers 2008), in this study we saw no such response.

In conclusion, endophyte infection in tall fescue affected soil P fractions, but had little significant influence on rhizosphere soil microbial community structure, function, or P uptake. The chemical form of P added, and its initial reaction with the soils prior to planting was the overriding factor influencing plant growth and rhizosphere biogeochemical processes. P deficiency led to an overall increase in potential AcPase activity and positive associations with AM fungi (irrespective of

endophyte status), which are typical plant mechanism used by most plants when confronted with P deficiency. Under the controlled conditions of this study, there is little support for endophyte enhanced acquisition of P from soils.

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