Microb Ecol (2016) 72:682–691
DOI 10.1007/s00248-016-0828-3

PLANT MICROBE INTERACTIONS

Aboveground *Epichloë coenophiala*–Grass Associations Do Not Affect Belowground Fungal Symbionts or Associated Plant, Soil Parameters

Lindsey C. Slaughter[1] · **Rebecca L. McCulley**[1]

Received: 8 June 2016 / Accepted: 27 July 2016 / Published online: 9 August 2016

Abstract Cool season grasses host multiple fungal symbionts, such as aboveground *Epichloë* endophytes and belowground arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSEs). Asexual *Epichloë* endophytes can influence root colonization by AMF, but the type of interaction—whether antagonistic or beneficial—varies. In *Schedonorus arundinaceus* (tall fescue), *Epichloë coenophiala* can negatively affect AMF, which may impact soil properties and ecosystem function. Within field plots of *S. arundinaceus* that were either *E. coenophiala*-free (E−), infected with the common, mammal-toxic *E. coenophiala* strain (CTE+), or infected with one of two novel, non-toxic strains (AR542 NTE+ and AR584 NTE+), we hypothesized that (1) CTE+ would decrease AMF and DSE colonization rates and reduce soil extraradical AMF hyphae compared to E− or NTE+, and (2) this would lead to E− and NTE+ plots having greater water stable soil aggregates and C than CTE+. *E. coenophiala* presence and strain did not significantly alter AMF or DSE colonization, nor did it affect extraradical AMF hypha length, soil aggregates, or aggregate-associated C and N. Soil extraradical AMF hypha length negatively correlated with root AMF colonization. Our results contrast with previous demonstrations that *E. coenophiala* symbiosis inhibits belowground AMF communities. In our mesic, relatively nutrient-rich grassland, *E. coenophiala* symbiosis did not antagonize belowground symbionts, regardless of strain. Manipulating *E. coenophiala* strains within *S. arundinaceus* may not significantly alter AMF communities and nutrient cycling, yet we must further explore these relationships under different soils and environmental conditions given that symbiont interactions can be important in determining ecosystem response to global change.

Keywords Arbuscular mycorrhizal fungi · Carbon sequestration · Dark septate endophytes · Grasslands · *Neotyphodium* · Tall fescue

Introduction

Plants form and maintain myriad symbioses with microorganisms. These relationships occur above- and belowground, can be host-specific, and function on a continuum of interactions from parasitism to mutualism. One symbiosis of great ecological importance occurs between *Schedonorus arundinaceus* (Schreb.) Dumort (tall fescue) (= *Lolium arundinaceum* (Schreb.) Darbysh. = *Festuca arundinacea* Schreb.), a cool-season grass, and *Epichloë coenophiala* (Morgan-Jones & W. Gams = *Neotyphodium coenophialum* (Morgan-Jones & W. Gams) = *Acremonium coenophialum* Morgan-Jones & W. Gams), an asexual (transmitted only via plant seeds) aboveground fungal endophyte thought to have evolved with *S. arundinaceus*. A large proportion of the 15 million hectares of *S. arundinaceus* across the USA [1], where it is non-native, is infected with *E. coenophiala* [2].

E. coenophiala is commonly a defensive mutualist, growing intercellularly within *S. arundinaceus* and consuming apoplastic sugars and amino acids. It enhances drought and mineral stress resistance [3–6], increases competitive ability

✉ Lindsey C. Slaughter
lincslau@gmail.com

[1] N-222N Agricultural Science Center North, Department of Plant and Soil Sciences, University of Kentucky, 1100 South Limestone, Lexington, KY 40546-0091, USA

[7], growth, and reproduction [8], and produces alkaloid compounds that deter herbivory [9, 10]. Consequently, *E. coenophiala* infection reduces plant diversity and gradually increases *S. arundinaceus* abundance in plant communities [11, 12]. Endophyte-infected (E+) *S. arundinaceus* stands also accumulate more soil organic carbon and total nitrogen with time compared to uninfected (E−) stands [13–15].

The most common strain of *E. coenophiala* in the USA produces ergot alkaloids that cause well-documented toxicity symptoms in grazing livestock, such as impaired heat tolerance and reduced reproductive success, which are cohesively termed "tall fescue toxicosis" [16]. Naturally occurring *E. coenophiala* strains that do not produce mammal-toxic ergot alkaloids, yet continue to deter insect herbivory through loline and peramine alkaloid production, have been isolated and introduced into forage cultivars [17], often reducing endophyte-conferred plant benefits [4]. These "novel" or "non-toxic" endophytes (NTEs) are increasingly present in managed grasslands worldwide, yet we do not fully understand the ecological implications of these symbioses on plant communities, soil properties, and concomitant symbionts in *S. arundinaceus*.

Another important plant-microbial symbiosis exists between nearly 80 % of land plants and arbuscular mycorrhizal fungi (AMF) of the phylum *Glomeromycota* [18, 19]. AMF colonize plant roots, increasing water and nutrient uptake in exchange for host photosynthate [19, 20]. The availability of nutrients, such as P and N, influences the relative benefit these nutritional mutualists confer to hosts. For example, AMF may be a parasitic sink for plant C when environmental N and P are in abundance, but become beneficial when P is limited [21]. AMF soil hyphal networks also improve soil physical properties such as aggregate size and stability, and increase C sequestration [22, 23].

Other belowground endophytic fungi frequently coexisting with AMF include dark septate endophytes (DSEs) of the phylum *Ascomycota*. DSE may perform similar or complementary functions to AMF, but researchers are just beginning to investigate these possibilities [24, 25]. If DSEs function similarly to AMF, both symbionts may influence plant productivity and soil properties in grassland ecosystems, such as *S. arundinaceus*-dominated pastures.

Little is known about how *S. arundinaceus*' aboveground symbiosis with *E. coenophiala* affects belowground symbioses with AMF and DSE. Symbiosis with CTE strains can decrease AMF root colonization rate in *S. arundinaceus* [26, 27]. CTE symbiosis also lowered the abundance of AMF spores [28] and lipid biomarker 16:1 ω5 cis [29] in soils compared to E−. Decomposing CTE+ thatch reduced AMF colonization rates in other plants, whereas E− and NTE+ (AR542 strain) did not [30]. This suggests that compounds produced in CTE+ *S. arundinaceus*, such as ergot alkaloids, negatively affect AMF. A similar asexual endophyte *Epichloë festucae* var. *lolii* can reduce AMF infection in *Lolium perenne* L. [31], yet Liu et al. [32] observed that competition between the endophyte and AMF was mitigated in a higher sugar host cultivar. *Epichloë occultans* (C.D. Moon, B. Scott & M.J. Chr.) [= *Neotyphodium occultans* C.D. Moon, B. Scott & M.J. Chr.] in *Lolium multiflorum* Lam. also decreased AMF colonization in E+ plants, but increased AMF colonization in neighboring E− plants [33]. Studies of other cool-season grasses hosting asexual *Epichloë* species show that endophyte infection may stimulate host AMF colonization [34–36] and augment plant growth [34, 37]. We do not fully understand the divergence between different grass host–endophyte–AMF relationships, nor have there been comprehensive examinations of these relationships considering *S. arundinaceus* containing different endophyte strains, DSE, or their impacts on related ecosystem parameters.

To address this knowledge gap, using plant and soil samples collected from a 5-year-old field study, we examined how CTE and NTE strains of *E. coenophiala* in *S. arundinaceus* affected root mycorrhizal and DSE colonization, associated shoot and root nutrients, lengths of soil extraradical AMF hyphae, water stable soil aggregates, and C and N within aggregates. We hypothesized that (1) CTE+ plots would decrease root AMF and DSE colonization rates and reduce extraradical AMF hyphae compared to E− or NTE+; and (2) these effects would lead to greater water stable soil aggregates and C concentration in E− and NTE+ plots than in CTE+.

Materials and Methods

Site Description and Study Design

The study was located in Lexington, Kentucky, at the University of Kentucky Spindletop Research Farm (38° 6′ 29″ N, 84° 29′ 31″ W), which has average summer and winter temperatures of 23.8 and 1.6 °C, respectively, and 1163 mm mean annual precipitation [38]. The soil is described as a Bluegrass-Maury silt loam weathered from a silty loess mantle over clayey phosphatic limestone residuum, and is a well-drained fine, mixed, semi-active, mesic Typic Paleudalf [39]. Soil C, N, and P levels at establishment (2008) were 2.25 % C, 0.25 % N, and 184 mg P kg^{-1} soil [12]. In May 2013, the mean soil test nutrient levels and Sikora II buffer pH measured by the University of Kentucky Soil Testing Regulatory Services were as follows: 184.21 mg P kg^{-1} soil, 90.81 mg K kg^{-1} soil, 1582.98 mg Ca kg^{-1} soil, 143.90 mg Mg kg^{-1} soil, 1.88 mg Zn kg^{-1} soil, and 6.57 pH.

On 10 April 2008, field plots were established in a randomized complete block design (RCBD) with six blocks containing four plots each, resulting in 24, 2 × 2 m total squares separated by 1 m *Poa pratensis* L. (Kentucky bluegrass) alleys. Pasture demonstration farm (PDF) variety *S. arundinaceus* seed was hand-

broadcast in monoculture at 11.2 kg ha^{-1} in each plot and contained one of four treatments: E−, infected with a CTE strain of *E. coenophiala* (CTE+), or infected with one of two NTE strains (AR542 NTE+ or AR584 NTE+). Endophyte frequency, via immunoblot assay, and endophyte strain, via genetic screening [40], were verified in May 2010. E− plots were 1 % infected, CTE+ plots were 84 % infected, AR542 NTE+ plots were 84 % infected, and AR584 NTE+ plots were 97 % infected. Genetic tests confirmed that CTE+ and NTE+ treatments were as planned. Tall fescue abundance (%) in treatment plots on 13 June 2013 averaged 51 ± 8 (E−), 92 ± 1 (CTE+), 64 ± 5 (AR542 NTE+), and 75 ± 6 (AR584 NTE+).

Sample Harvest and Preparation

Five years after establishment (30 May 2013), we harvested ramets (2–4 vegetative tillers) of *S. arundinaceus* with intact roots from three plants within each plot, obtaining 72 total samples. Three 1.5 cm diameter soil cores were collected and composited for each plot, totaling 24 soil samples, which were sieved to 2 mm and air-dried. Roots were separated from each ramet, washed, and dried at 55 °C. After AMF and DSE colonization analysis, composited roots per plot were cyclone milled. Endophyte presence/absence was verified in individual tillers within each ramet using an *Epichloë*-specific immunoblot assay [41]. Tillers comprising six ramets tested as E− in CTE+ or NTE+ treatments out of the 72 samples and were excluded from the study. Tillers were composited per plot, dried, and milled.

Root Mycorrhizal and DSE Colonization

Mycorrhizal and DSE colonization in *S. arundinaceus* were measured via root microscopy [42]. Dried root subsections were cleared using 10 % KOH, acidified in 2 % HCl, and stained with 0.05 % trypan blue. Roots were de-stained in 1:1 glycerol/deionized (DI) water, then allowed to dry on 25-mm microscope slides before preserving with polyvinyl lactoglycerol (PVLG; INVAM). AMF colonization rate was measured using a line intersect method at ×400 magnification modified from McGonigle et al. [42], counting the presence of AMF arbuscules, vesicles, or hyphae at each intersection. Only one count was recorded when structures intersected, prioritizing arbuscules > vesicles > hyphae. We also tallied melanized, septate DSE hyphae or microsclerotia presence. Total AMF and DSE colonization (%) was calculated as the number of presences divided by possible views and multiplied by 100.

Plant Nutrients

Total N and P concentrations within milled root and shoot tissue were measured via wet digestion. Plant N was converted to NH_3 [43] and colorimetrically determined (%) via modified Berthelot reaction [44]. Plant P was reduced to PO_4^{3-} and colorimetrically determined (%) based on Fiske and Subbarow [45].

Extraradical AMF in Soil

To estimate the length of extraradical AMF hyphae, we extracted hyphae from a 4-g soil subsample by an aqueous extraction and membrane filter technique modified from Jakobsen et al. [46] and Rillig et al. [47]. Subsamples were dispersed in 100 mL of DI water with an added 12 mL of 35 g L^{-1} $(NaPO_3)_6$. Solutions were shaken by hand, sonicated, allowed to settle before passing through a 38-μm sieve to retain hyphae, roots, and organic material, and then washed into 250-mL flasks with 200 mL of DI water. A 4-mL aliquot was pipetted into a syringe attached to a 25-mm Millipore filter holder containing 0.45-μm pore size nitrocellulose membrane filters. Aliquots were stained with 0.05 % trypan blue for 1.5 h [48], vacuum-filtered to retain stained AMF hyphae on the membrane, and rinsed with DI water. Membranes were allowed to dry on 25-mm microscope slides before preserving with PVLG. We estimated AMF hyphal length via gridline-intersect method [48] with a 10-mm^2 gridded graticule (100 squares total) at ×100 magnification and 50 fields of view per slide, differentiating AMF and non-AMF hyphae using criteria for internal hyphae [49–51]. Extraradical AMF hypha length within each plot was calculated as m hyphae g^{-1} soil, using Tennant's equation [48]. Extraction efficiency was measured as described in Miller et al. [49], resulting in a correction factor of 88 % efficiency.

Soil Aggregate Stability and Nutrients

We determined water-stable soil aggregate percentage in soils using wet-sieving apparatus (Eijkelkamp, Giesbeek, NL) as described in Wuddivira and Camps-Roach [52]. A 4-g soil subsample from each plot was placed into the apparatus equipped with a 250-μm sieve (small macroaggregates), covered with DI water, and rotary sieved for 3 min (stroke = 1.3 cm, approximately 34 times/min). All material washed through the 250-μm sieve was passed through a 53-μm sieve (microaggregates). Material retained on each sieve was dispersed by sieving in a solution of 2 g L^{-1} $(NaPO_3)_6$ for 5–8 min. The dispersed solutions from each sieve size and the material not retained on sieves (not water stable, NWS) were transferred into pre-weighed cans and dried at 105 °C for 48 h. Percentage water-stable small macroaggregates (250–2000 μm) and microaggregates (53–250 μm) within each sample were calculated using the weight of soil obtained in the dispersing solution cans for each sieve size divided by the sum weight obtained in both dispersing solution cans and the distilled water can. Total C and N

concentrations (%) within dried, ball-ground soils from each aggregate fraction were determined on an elemental analyzer (FlashEA 1112 series, Thermo Fisher Scientific, Waltham, MA).

Statistical Analysis

Significant main effects of endophyte treatment (α = 0.05) were assessed on AMF and DSE root colonization rate, tissue N and P for roots and shoots, and percentage of water stable soil macro- and microaggregates using the PROC MIXED procedure in SAS (9.3 SAS Institute Inc., Cary, NC, USA) for a RCBD design with endophyte treatment as a fixed effect and block as a random effect. Averaged AMF and DSE colonization rates across individual ramets per plot were used for statistical analysis. Significant fixed endophyte treatment and soil aggregate size fraction effects on aggregate C and N were analyzed as a split-plot design using PROC MIXED, with block as a random effect. Significant differences between means were compared using LSMEANS and the PDIFF option in SAS. Potential correlations between quantitative parameters such as AMF colonization and plant nutrients were explored using the PROC REG procedure in SAS and are reported where significant.

Results

Root Mycorrhizal and DSE Colonization

Total AMF and DSE colonization in *S. arundinaceus* roots at the site averaged 38 (±2) and 20 (±1) %, respectively. *E. coenophiala* treatment did not significantly affect total root AMF colonization rates (%) (Fig. 1a; P = 0.5751), although CTE+ plants had lower AMF colonization than E− or NTE+ treatments (33 % in CTE+ vs. 40 % averaged across E−, NTE+). Endophyte treatment did not significantly affect AMF arbuscule, vesicle, or hypha presence ($F_{3,\ 15}$ = 0.42–1.43; all P > 0.05) or DSE colonization ($F_{3,\ 15}$ = 0.10; P = 0.9586; Table 1). We also observed no correlation between total AMF and DSE colonization (regression P = 0.8313; R^2 = 0.0021).

Plant Nutrients

Endophyte treatment did not significantly influence %N ($F_{3,\ 15}$ = 1.13; P = 0.3669) or P ($F_{3,\ 15}$ = 1.04; P = 0.4025) in root tissue (Fig. 2a, b), or shoot tissue N ($F_{3,\ 15}$ = 2.86; P = 0.0722) or P ($F_{3,\ 15}$ = 0.38; P = 0.7672; Fig. 2a, b). However, shoot %N trended greater in CTE+ compared to E− (LSMeans p = 0.0112, CTE+ 0.16 percentage points > E−). Additionally, plant shoot %P significantly correlated with total AMF colonization, where %AMF was higher in plants containing lower shoot %P (Fig. 3). No other significant nutritional relationships were identified. There was no

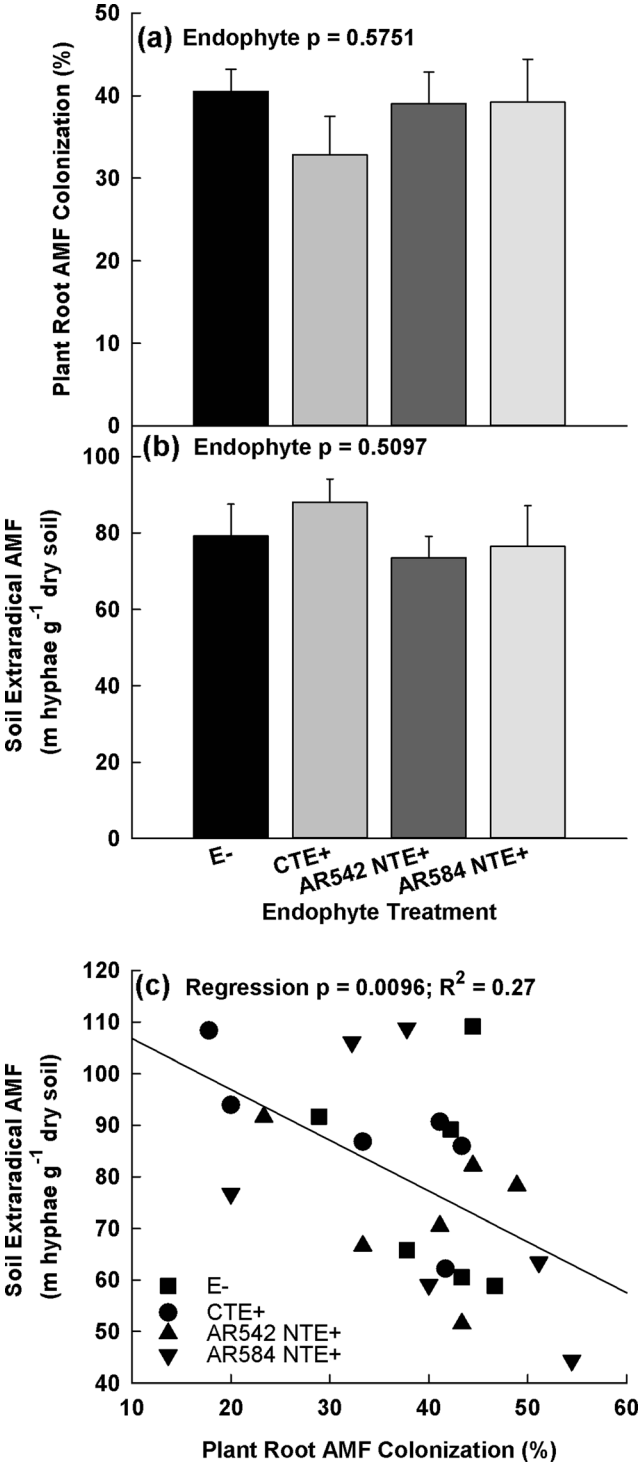

Fig. 1 **a** Root colonization rate (%) of arbuscular mycorrhizal fungi (AMF) measured in *S. arundinaceus* roots. **b** Length of extraradical AMF hyphae in soil samples (m hyphae g^{-1} dry soil). Values in **a** and **b** are means (±S.E.) of the 6 replicates within each treatment, while **c** shows the linear regression of soil extraradical AMF (m hyphae g^{-1} dry soil) with *S. arundinaceus* root AMF colonization (%) across the 24 research plots labeled by endophyte treatment

Table 1 Colonization rates (%) of arbuscular mycorrhizal fungi (AMF) arbuscules, vesicles, and hyphae, and the rate of dark septate endophyte (DSE) colonization (total hyphae and microsclerotia) measured in *S. arundinaceus* roots. Values are means (±S.E.) of 6 replicates within each treatment

	Endophyte treatment				
	E−	CTE+	AR542 NTE+	AR584 NTE+	Site average
AMF arbuscules (%)	11 (2)	8 (3)	14 (3)	15 (3)	12 (1)
AMF vesicles (%)	5 (1)	5 (1)	6 (1)	3 (1)	5 (1)
AMF hyphae (%)	24 (3)	20 (4)	19 (2)	21 (4)	21 (2)
DSE colonization (%)	19 (3)	21 (2)	20 (3)	19 (3)	20 (1)

endophyte treatment effect on plant N/P ratio in roots ($F_{3,\ 15}$ = 0.23; P = 0. 0.8722) or shoots ($F_{3,\ 15}$ = 0.67; P = 0.5823; Table 2).

Extraradical Soil AMF

Endophyte treatment did not significantly affect soil extraradical AMF hyphal length (m hyphae g^{-1} soil; Fig. 1b; $F_{3,\ 15}$ = 0.81; P = 0.5097). Unlike root colonization, CTE+ plots typically exhibited greater soil hyphal length than E− or NTE+ plots (particularly AR542 NTE+). Plots with greater total root AMF colonization generally contained less extraradical soil hyphae (Fig. 1c), but we found no significant plant or soil correlations.

Soil Aggregate Stability and Nutrients

Endophyte treatment did not significantly influence the proportion of NWS aggregates ($F_{3,\ 15}$ = 1.30; P = 0.3109), water stable microaggregates 53–250 μm ($F_{3,\ 15}$ = 0.45; P = 0.7221), water stable small macroaggregates 250–2000 μm ($F_{3,\ 15}$ = 0.64; P = 0.5992), or total water stable aggregate amount (macro + micro; $F_{3,\ 15}$ = 1.30; P = 0.3109).

Soil aggregate C was determined by size (aggregate size $F_{2,\ 40}$ = 45.74; P = <0.0001), but not endophyte treatment (endophyte $F_{3,\ 15}$ = 1.89; P = 0.1755) or the interaction between aggregate size and endophyte treatment (endophyte * aggregate size $F_{6,\ 40}$ = 1.11; P = 0.3758). Carbon was highest in small macroaggregate and NWS fractions but not different between them (Table 3). Soil aggregate N was also determined by size (aggregate size $F_{2,\ 40}$ = 116.24; P = <0.0001), and not endophyte treatment (endophyte $F_{3,\ 15}$ = 0.92; P = 0.4548) or the interaction (endophyte * aggregate size $F_{6,\ 40}$ = 0.47; P = 0.8241). N

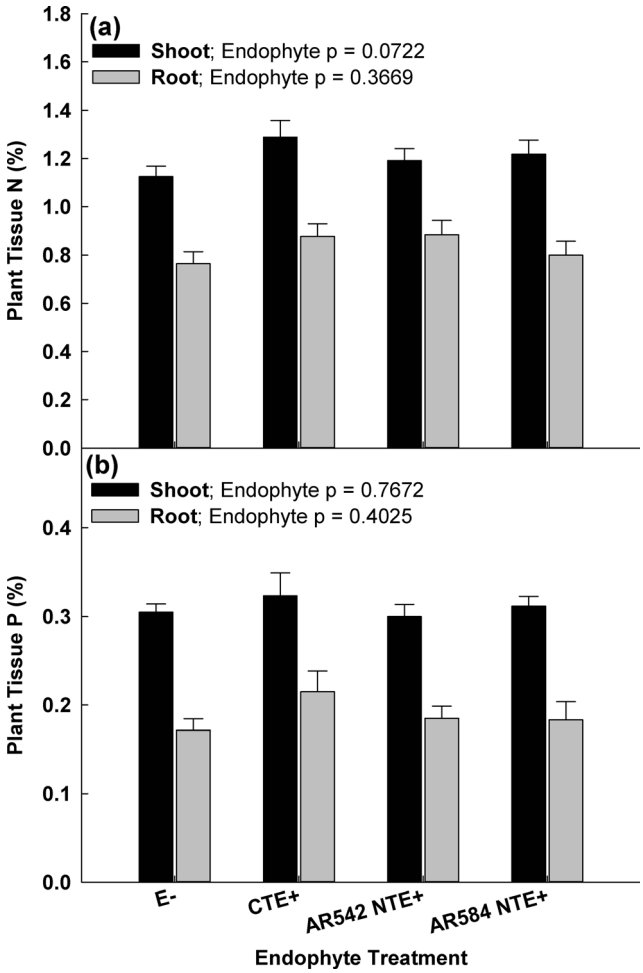

Fig. 2 **a** N and **b** P concentration (%) in *S. arundinaceus* tissue. Values are means (±S.E.) of each treatment

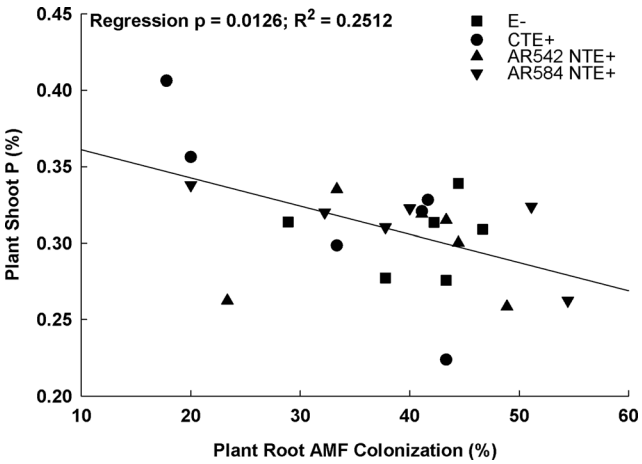

Fig. 3 Linear regression of shoot P concentration (%) and root AMF colonization (%) in *S. arundinaceus*

Table 2 Ratio of N/P in *S. arundinaceus* tissue. Values are means (±S.E.)

	Endophyte treatment				
N/P	E−	CTE+	AR542 NTE+	AR584 NTE+	Site average
Shoot	3.72 (0.23)	4.04 (0.15)	4.01 (0.16)	3.92 (0.25)	3.92 (0.10)
Root	4.55 (0.28)	4.36 (0.53)	4.78 (0.25)	4.52 (0.30)	4.55 (0.17)

concentration was also highest in small macroaggregate and NWS fractions but not different between them (Table 3).

Discussion

To our knowledge, this is the first study quantifying aboveground *E. coenophiala* strain effects on root AMF and DSE colonization and soil hyphae. None of these characteristics differed between E−, CTE+, AR542 NTE+, and AR584 NTE+ plants. There were no significant symbiont-associated changes in above- or belowground plant nutrients, soil aggregates, or aggregate-associated C and N, although we observed negative correlations between plant shoot P and root AMF colonization, and between soil and root AMF. Aboveground *E. coenophiala*, regardless of strain or alkaloid production potential, may neither antagonize belowground AMF and DSE in shared *S. arundinaceus* hosts nor substantially affect plant nutrients or soil properties in mesic, P-rich temperate-managed grasslands.

Our first hypothesis that CTE symbiosis in *S. arundinaceus* would inhibit root AMF and DSE colonization compared to NTE+ or E− plants was unsupported by this study. This contrasts with previous demonstrations that CTE symbiosis reduces AMF colonization in roots and inhibits AMF propagules in soils [26–28], potentially due to methodological and environmental differences. Prior studies lasted one growing season (103 days from seed in Mack and Rudgers [27], 15 weeks from seed in Guo et al. [26]) and used either a live soil inoculum from nearby fields, a commercial fungal inoculum with one strain [27], or single-species isolates of *Glomus* sp. taken from field soils [26]. Chu-Chou et al. [28] used soil and *S. arundinaceus* seeds harvested from 3-year-old field plots of CTE+ and E− plants, and measured propagules kilograms per soil and spores per plant using most probable number (MPN) assays [53, 54]. By serially diluting soil samples with sterilized sand, growing host plants from seed, then harvesting soil and roots to examine spores and AMF propagules, this method essentially captures initial seedling colonization capacity using environmental inoculum. Our direct assessment of plants and soils from 5-year-old field plots confounds equitable comparison to these studies.

S. arundinaceus in this study experienced 5 growing seasons, and only *E. coenophiala* within seeds was manipulated, with no controls on soil microbes. Plants harboring different *E. coenophiala* strains may have accumulated different

Table 3 Percentage (%) of non-water stable (NWS) silt and clay, water stable microaggregates (53–250 μm), and water stable small macroaggregates (250–2000 μm) in soil samples, and aggregate-associated C and N concentration (%). Values are means (±S.E.)

	Endophyte treatment				
Aggregate size	E−	CTE+	AR542 NTE+	AR584 NTE+	Site average
% NWS[a]	9.8 (0.3)	11.0 (1.0)	11.3 (0.4)	10.0 (0.9)	10.2 (0.3)
% C	2.1 (0.0)	2.1 (0.0)	2.2 (0.0)	2.3 (0.1)	2.2 (0.0)
% N	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.3 (0.0)	0.2 (0.0)
% 53–250 μm[b]	12.5 (1.4)	11.0 (1.0)	12.3 (1.6)	11.0 (1.0)	11.7 (0.6)
% C	1.7 (0.0)	1.7 (0.1)	1.9 (0.1)	1.7 (0.1)	1.8 (0.1)
% N	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)
% 250–2000 μm[a]	77.7 (1.5)	79.2 (1.7)	76.4 (1.9)	79.1 (1.5)	78.1 (0.9)
% C	2.2 (0.0)	2.2 (0.0)	2.2 (0.0)	2.3 (0.0)	2.2 (0.0)
% N	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.3 (0.0)	0.2 (0.0)

[a, b] Different letters indicate significantly different mean C and N between soil aggregate sizes ($F_{2, 40}$ = 45.74–116.24; all aggregate size P = <0.0001). Although C and N were analyzed individually, one letter is used to indicate parallel patterns of significant differences between soil aggregate sizes for ease of interpretation

belowground fungal communities with time [55]. While prior studies proved that *E. coenophiala* symbiosis can reduce root AMF colonization in *S. arundinaceus* and inhibit certain AMF species in surrounding soils, we find these differences may not persist with time or in certain field conditions, or extend to other belowground symbionts such as DSE. Research remains to be done evaluating potential effects of different endophyte strains on the establishment and composition of belowground fungal communities over time.

Neither endophyte treatment nor root symbiont colonization rates significantly determined plant nutrient concentrations. Average plant root and shoot N/P ratios of 4.6 and 3.9, respectively, indicate a relatively N-limited, P-rich site [56], which predictive theory suggests would foster a commensal plant–AMF relationship [21]. We noted a weak negative correlation between AMF colonization and shoot P (Fig. 3). This was also observed in Ryan et al. [57], perhaps suggesting that plants relied more on AMF colonization as they became P-deficient in shoots. Alternatively, in this site's high P soils, the optimum plant benefit from AMF was achieved at lower colonization rates while higher colonization rates produced no additional benefit or even antagonistic feedback to plant P [58]. Because we cannot fully evaluate AMF contribution to P uptake at different colonization levels based solely on plant P [59], we cannot completely delineate this relationship. Potential commensalism between *S. arundinaceus* and AMF at our P-rich site may have modulated nutritional contribution from AMF, and thus potentially interactions between *E. coenophiala* and AMF, which would likely have differed under nutrient limitation.

We had further hypothesized that plots containing CTE+ *S. arundinaceus* would support less extraradical AMF hyphae compared to NTE+ or E− plots, which was also not validated by our study results. We observed the opposite trend, with a small increase in the length of extraradical soil hyphae in CTE+ plots compared to E− or the two NTE+ treatments. This contrasts with Antunes et al. [30], where root AMF colonization was inhibited in *Bromus inermis* Leyss. (smooth brome) subjected for 120 days to decomposing CTE+ *S. arundinaceus* thatch, but not to AR542 NTE+ thatch, suggesting that extraradical growth through soil may have been affected. Although lacking significant differences due to endophyte presence, our results support Antunes et al.'s [30] alternative hypothesis: differences between CTE+ and NTE+ strains are not due specifically to the presence or amounts of livestock-toxic ergot alkaloids, but to other differences dependent on host genetics or nutrient resources, such as other alkaloids or metabolites [60, 61], or root exudates [62].

These data also contrast with those from an adjacent experiment of a similar age containing the same *S. arundinaceus* variety and endophyte treatments, in which Rojas et al. [63] found increased AMF DNA abundance in bulk and rhizosphere soils of E+ plots compared to E− regardless of endophyte strain. Uneven distribution of nuclei within aseptate AMF hyphae can unbalance analyses of DNA abundance and cause poor correlation with microscopy-based examinations [64] such as estimates of extraradical hyphal length. Further, certain AMF species preferentially produce either spores, hyphae, or root colonization structures [65], and AMF spores and hyphae harbor different concentrations of nuclei [66]. We evaluated hyphal length via microscopy and did not account for spores present in soil samples which would have been included in DNA analyses, which may explain why our results differed from Rojas et al. [63].

Species-specific allocation between fungal structures also cause differences in AMF communities between roots and soil extraradical mycelium [67, 68], which may explain why plant root AMF was inversely proportional to the amount of extraradical soil AMF in this study (Fig. 1c). Although not statistically significant, it is possible that endophyte-mediated effects on AMF could manifest as tradeoffs between root colonization and soil networks, as suggested by lower root colonization rates but higher extraradical soil hyphae in CTE+ stands. These tradeoffs could intensify with reduced plant diversity and increased abundance of CTE+ *S. arundinaceus* compared to E− or NTE+ plots. Although AMF and DSE can be positively correlated [69], lack of an *E. coenophiala* effect or correlation between AMF and DSE in this study obfuscates hypotheses regarding these symbionts. Future studies of *Epichloë* endophyte effects on belowground symbioses within hosts and soil should consider differences in fungal species.

Our second hypothesis that long-term E− or NTE+ field plots supporting increased soil hyphae would have more water stable soil aggregates was also not validated by our study. This is likely because we found no significant changes in extraradical soil hyphae, which impact soil aggregate size [23]. Our lack of observed changes in both macro- and microaggregates, and C and N within aggregates contrasts with a prior study finding that C and N was primarily accumulated and protected in small macroaggregates due to CTE symbiosis [14]. Stand age might have contributed to our results, as endophyte effects on soil aggregates or aggregate-associated C or N can be difficult to detect in short-term studies (e.g., ≤60 weeks [70, 71]), yet have been detected after at least 5, 8, or 20 years [13–15]. Because our analysis occurred 5 years after planting, increases in extraradical hyphae within CTE+ plots in the current study, even with greater CTE+ *S. arundinaceus* abundance, may not have had sufficient time to significantly impact soil aggregates and C sequestration.

Conclusions

In this 5-year-old field study, neither *E. coenophiala* presence nor strain significantly impacted root AMF or DSE

colonization of *S. arundinaceus* or soil extraradical hyphae. CTE+ plots exhibited lower root AMF colonization but greater extraradical hyphae length compared to other treatments, but these subtle effects did not cause any endophyte-associated changes on plant P or N, soil aggregates, or aggregate C or N. Our report of similar soil AMF and root AMF and DSE within two strains of NTE+ *S. arundinaceus* is novel, and the disparity of our results with those of prior studies examining *E. coenophiala*/*S. arundinaceus*/AMF relationships call attention to the sensitivity of these tripartite interactions to other environmental parameters, such as stand age, field conditions, and AMF species. We suggest that presence or manipulation of *E. coenophiala* strains in *S. arundinaceus* may not substantially alter belowground symbioses and associated plant nutrition or nutrient cycling, at least in P-rich grasslands in the USA. High soil P may have promoted commensal interactions between aboveground *E. coenophiala* and belowground AMF and DSE. Future studies should mechanistically explore how these effects could differ among other soils or field conditions, considering the potential for interactions between symbionts (or lack thereof) to impact resource management decisions, ecosystem properties, and ecosystem response to global change factors.

Acknowledgments Lindsey C. Slaughter was supported by the UK Department of Plant and Soil Sciences. The authors thank J. Nelson and E. Carlisle for field maintenance and assistance, J. Crutchfield for plant N and P analysis, and K. Jacobsen for soil aggregate analysis equipment. We thank Sarah Hall (Berea College) for training in AMF colonization, and Dan Weber and Eric Kalosa-Kenyon for harvest assistance. We appreciate the Noble Foundation for providing *S. arundinaceus* seed and endophyte genetic assessments, and UK Regulatory Services Soil Testing Lab for soil nutrient characterization. This field project was supported by the Kentucky Agricultural Experiment Station (KY006045) and a cooperative agreement between UK's College of Agriculture, Food, and the Environment and the USDA-ARS-Forage Animal Production Research Unit (Award No. 58-6440-7-135).

References

1. Rogers JK, Locke JM (2013) Tall fescue: history, application, establishment and management. Agricultural Publications. The Samuel Roberts Noble Foundation, Ardmore, pp. 1–8
2. Shelby RA, Dalrymple LW (1987) Incidence and distribution of the tall fescue endophyte in the United States. Plant Dis. 71:783–786. doi:10.1094/Pd-71-0783
3. Arachevaleta M, Bacon CW, Hoveland CS, Radcliffe DE (1989) Effect of the tall fescue endophyte on plant response to environmental stress. Agron. J. 81:83–90. doi:10.2134/agronj1989.00021962008100010015x
4. Bouton JH, Gates RN, Belesky DP, Owsley M (1993) Yield and persistence of tall fescue in the southeastern coastal plain after removal of its endophyte. Agron. J. 85:52–55. doi:10.2134/agronj1993.00021962008500010011x
5. Elmi AA, West CP (1995) Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. New Phytol 131:61–67. doi:10.1111/j.1469-8137.1995.tb03055.x
6. Malinowski DP, Alloush GA, Belesky DP (2000) Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. Plant Soil 227:115–126. doi:10.1023/A:1026518828237
7. Hill NS, Belesky DP, Stringer WC (1991) Competitiveness of tall fescue as influenced by *Acremonium coenophialum*. Crop Sci. 31:185–190. doi:10.2135/cropsci1991.0011183X003100010042x
8. Gundel PE, Helander M, Casas C, Hamilton CE, Faeth SH, Saikkonen K (2013) *Neotyphodium* fungal endophyte in tall fescue (*Schedonorus phoenix*): a comparison of three northern European wild populations and the cultivar Kentuky-31. Fungal Divers. 60:15–24. doi:10.1007/s13225-012-0173-x
9. Bush LP, Fannin FF, Siegel MR, Dahlman DL, Burton HR (1993) Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte-grass interactions. Agric. Ecosyst. Environ. 44:81–102. doi:10.1016/0167-8809(93)90040-V
10. Porter JK, Bacon CW, Robbins JD, Betowski D (1981) Ergot alkaloid identification in clavicipitaceae systemic fungi of pasture grasses. J. Agric. Food Chem. 29:653–657. doi:10.1021/jf00105a055
11. Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. Science 285:1742–1744. doi:10.1126/science.285.5434.1742
12. Iqbal J, Nelson JA, McCulley RL (2013) Fungal endophyte presence and genotype affect plant diversity and soil-to-atmosphere trace gas fluxes. Plant Soil 364:15–27. doi:10.1007/s11104-012-1326-0
13. Franzluebbers AJ, Nazih N, Stuedemann JA, Fuhrmann JJ, Schomberg HH, Hartel PG (1999) Soil carbon and nitrogen pools under low- and high-endophyte-infected tall fescue. Soil Sci. Soc. Am. J. 63:1687–1694
14. Franzluebbers AJ, Stuedemann JA (2005) Soil carbon and nitrogen pools in response to tall fescue endophyte infection, fertilization, and cultivar. Soil Sci. Soc. Am. J. 69:396–403. doi:10.2136/sssaj2005.0396
15. Iqbal J, Siegrist JA, Nelson JA, McCulley RL (2012) Fungal endophyte infection increases carbon sequestration potential of southeastern USA tall fescue stands. Soil Biol. Biochem. 44:81–92. doi:10.1016/j.soilbio.2011.09.010
16. Strickland JR, Looper ML, Matthews JC, Rosenkrans CF, Flythe MD, Brown KR (2011) St. Anthony's fire in livestock: causes, mechanisms, and potential solutions. J. Anim. Sci. 89:1603–1626. doi:10.2527/jas.2010-3478
17. Bouton JH, Latch GCM, Hill NS, Hoveland CS, McCann MA, Watson RH, Parish JA, Hawkins LL, Thompson FN (2002) Reinfection of tall fescue cultivars with non-ergot alkaloid–producing endophytes. Agron. J. 94:567–574. doi:10.2134/agronj2002.5670
18. Schüβler A, Schwarzott D, Walker C (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol. Res. 105:1413–1421. doi:10.1017/s0953756201005196
19. Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Academic Press, London
20. Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42. doi:10.1007/s005720100097
21. Johnson NC (2010) Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. New Phytol 185:631–647. doi:10.1111/j.1469-8137.2009.03110.x
22. Duchicela J, Sullivan TS, Bontti E, Bever JD (2013) Soil aggregate stability increase is strongly related to fungal community succession along an abandoned agricultural field chronosequence in the Bolivian Altiplano. J. Appl. Ecol. 50:1266–1273. doi:10.1111/1365-2664.12130
23. Miller RM, Jastrow JD (1990) Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. Soil Biol. Biochem. 22:579–584. doi:10.1016/0038-0717(90)90001-G

24. Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. Stud. Mycol. 53: 173–189. doi:10.3114/sim.53.1.173
25. Mandyam KG, Jumpponen A (2014) Mutualism–parasitism paradigm synthesized from results of root-endophyte models. Front. Microbiol. 5:776. doi:10.3389/fmicb.2014.00776
26. Guo BZ, Hendrix JW, An ZQ, Ferriss RS (1992) Role of *Acremonium* endophyte of fescue on inhibition of colonization and reproduction of mycorrhizal fungi. Mycologia 84:882–885. doi:10.2307/3760286
27. Mack KML, Rudgers JA (2008) Balancing multiple mutualists: asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes. Oikos 117:310–320. doi:10.1111/j.2007.0030-1299.15973.x
28. Chu-Chou M, Guo B, An ZQ, Hendrix JW, Ferriss RS, Siegel MR, Dougherty CT, Burrus PB (1992) Suppression of mycorrhizal fungi in fescue by the *Acremonium coenophialum* endophyte. Soil Biol. Biochem. 24:633–637. doi:10.1016/0038-0717(92)90041-u
29. Buyer JS, Zuberer DA, Nichols KA, Franzluebbers AJ (2011) Soil microbial community function, structure, and glomalin in response to tall fescue endophyte infection. Plant Soil 339:401–412. doi:10.1007/s11104-010-0592-y
30. Antunes PM, Miller J, Carvalho LM, Klironomos JN, Newman JA (2008) Even after death the endophytic fungus of *Schedonorus phoenix* reduces the arbuscular mycorrhizas of other plants. Funct. Ecol. 22:912–918. doi:10.1111/j.1365-2435.2008.01432.x
31. Müller J (2003) Artificial infection by endophytes affects growth and mycorrhizal colonisation of *Lolium perenne*. Funct. Plant Biol. 30:419–424. doi:10.1071/FP02189
32. Liu Q, Parsons AJ, Xue H, Fraser K, Ryan GD, Newman JA, Rasmussen S (2011) Competition between foliar *Neotyphodium lolii* endophytes and mycorrhizal *Glomus* spp. fungi in *Lolium perenne* depends on resource supply and host carbohydrate content. Funct. Ecol. 25:910–920. doi:10.1111/j.1365-2435.2011.01853.x
33. Omacini M, Eggers T, Bonkowski M, Gange AC, Jones TH (2006) Leaf endophytes affect mycorrhizal status and growth of co-infected and neighbouring plants. Funct. Ecol. 20:226–232. doi:10.1111/j.1365-2435.2006.01099.x
34. Novas MV, Cabral D, Godeas AM (2005) Interaction between grass endophytes and mycorrhizas in *Bromus setifolius* from Patagonia, Argentina. Symbiosis 40:23–30
35. Novas MV, Iannone LJ, Godeas AM, Cabral D (2009) Positive association between mycorrhiza and foliar endophytes in *Poa bonariensis*, a native grass. Mycol. Prog. 8:75–81. doi:10.1007/s11557-008-0579-8
36. Novas MV, Iannone LJ, Godeas AM, Scervino JM (2011) Evidence for leaf endophyte regulation of root symbionts: effect of *Neotyphodium* endophytes on the pre-infective state of mycorrhizal fungi. Symbiosis 55:19–28. doi:10.1007/s13199-011-0140-4
37. Larimer AL, Bever JD, Clay K (2012) Consequences of simultaneous interactions of fungal endophytes and arbuscular mycorrhizal fungi with a shared host grass. Oikos 121:2090–2096. doi:10.1111/j.1600-0706.2012.20153.x
38. Ferreira WPM, Priddy TK, Souza CF, Matthews J (2010) Trends in precipitation and air temperature time series in Lexington, KY-USA. ASABE, Annual International Meeting, Pittsburgh, Pennsylvania.
39. Soil Survey Staff et al. (2014) Web soil survey. Natural Resources Conservation Service, United States Department of Agriculture. http://websoilsurvey.nrcs.usda.gov/. Accessed 9 October 2015
40. Takach JE, Young CA (2014) Alkaloid genotype diversity of tall fescue endophytes. Crop Sci. 54:667–678. doi:10.2135/cropsci2013.06.0423
41. Hiatt EE, Hill NS, Bouton JH, Stuedemann JA (1999) Tall fescue endophyte detection: commercial immunoblot test kit compared with microscopic analysis. Crop Sci. 39:796–799. doi:10.2135/cropsci1999.0011183X003900030030x
42. McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular—arbuscular mycorrhizal fungi. New Phytol 115:495–501. doi:10.1111/j.1469-8137.1990.tb00476.x
43. Bradstreet RB (1965) Chapter III—digestion procedure. In: Bradstreet, RB (ed.) The Kjeldahl method for organic nitrogen. Academic Press, pp. 89–145
44. Chaney AL, Marbach EP (1962) Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130–132
45. Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66:375–400
46. Jakobsen I, Abbott LK, Robson AD (1992) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. New Phytol 120:371–380. doi:10.1111/j.1469-8137.1992.tb01077.x
47. Rillig MC, Field CB, Allen MF (1999) Soil biota responses to long-term atmospheric CO_2 enrichment in two California annual grasslands. Oecologia 119:572–577. doi:10.1007/s004420050821
48. Brundrett M, Addy H, McGonigle T (1994) Chapter 2: extracting, staining and measuring hyphae from soil. In: Brundrett M, Melville L, Peterson L (eds) Practical methods in mycorrhizal research. Mycologue Publications Ltd., Waterloo, Ontario
49. Miller RM, Jastrow JD, Reinhardt DR (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. Oecologia 103:17–23. doi:10.1007/BF00328420
50. Mosse B (1959) Observations on the extra-matrical mycelium of a vesicular-arbuscular endophyte. Trans. Br. Mycol. Soc. 42:439–IN435. doi:10.1016/S0007-1536(59)80044-9
51. Nicolson TH (1959) Mycorrhiza in the Gramineae: I. Vesicular-arbuscular endophytes, with special reference to the external phase. Trans. Br. Mycol. Soc. 42:421–IN423. doi:10.1016/S0007-1536(59)80043-7
52. Wuddivira MN, Camps-Roach G (2007) Effects of organic matter and calcium on soil structural stability. Eur. J. Soil Sci. 58:722–727. doi:10.1111/j.1365-2389.2006.00861.x
53. McGraw AC, Hendrix J (1986) Influence of soil fumigation and source of strawberry plants on population densities of spores and infective propagules of endogonaceous mycorrhizal fungi. Plant Soil 94:425–434. doi:10.1007/BF02374335
54. An ZQ, Hendrix JW, Hershman DE, Henson GT (1990) Evaluation of the "most probable number" (MPN) and wet-sieving methods for determining soil-borne populations of endogonaceous mycorrhizal fungi. Mycologia 82:576–581. doi:10.2307/3760048
55. Vandenkoornhuyse P, Ridgway KP, Watson IJ, Fitter AH, Young JPW (2003) Co-existing grass species have distinctive arbuscular mycorrhizal communities. Mol. Ecol. 12:3085–3095. doi:10.1046/j.1365-294X.2003.01967.x
56. Koerselman W, Meuleman AFM (1996) The vegetation N:P ratio: a new tool to detect the nature of nutrient limitation. J. Appl. Ecol. 33: 1441–1450. doi:10.2307/2404783
57. Ryan MH, Small DR, Ash JE (2000) Phosphorus controls the level of colonisation by arbuscular mycorrhizal fungi in conventional and biodynamic irrigated dairy pastures. Aust. J. Exp. Agric. 40:663–670. doi:10.1071/EA99005
58. Gange AC, Ayres RL (1999) On the relation between arbuscular mycorrhizal colonization and plant 'benefit'. Oikos 87:615–621. doi:10.2307/3546829
59. Smith SE, Smith FA, Jakobsen I (2004) Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol 162:511–524. doi:10.1111/j.1469-8137.2004.01039.x

60. Rasmussen S, Parsons AJ, Bassett S, Christensen MJ, Hume DE, Johnson LJ, Johnson RD, Simpson WR, Stacke C, Voisey CR, Xue H, Newman JA (2007) High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. New Phytol 173:787–797. doi:10.1111/j.1469-8137.2006.01960.x
61. Rasmussen S, Parsons AJ, Fraser K, Xue H, Newman JA (2008) Metabolic profiles of *Lolium perenne* are differentially affected by nitrogen supply, carbohydrate content, and fungal endophyte infection. Plant Physiol. 146:1440–1453. doi:10.1104/pp.107.111898
62. Guo J, McCulley RL, McNear DH (2015) Tall fescue cultivar and fungal endophyte combinations influence plant growth and root exudate composition. Front Plant Sci 6:183. doi:10.3389/fpls.2015.00183
63. Rojas X, Guo J, Leff JW, McNear DH, Fierer N, McCulley RL (2016) Infection with a shoot-specific fungal endophyte (*Epichloë*) alters tall fescue soil microbial communities. Microb. Ecol.:1–10. doi:10.1007/s00248-016-0750-8
64. Gamper HA, Young JPW, Jones DL, Hodge A (2008) Real-time PCR and microscopy: are the two methods measuring the same unit of arbuscular mycorrhizal fungal abundance? Fungal Genet. Biol. 45:581–596. doi:10.1016/j.fgb.2007.09.007
65. Varela-Cervero S, Vasar M, Davison J, Barea JM, Opik M, Azcon-Aguilar C (2015) The composition of arbuscular mycorrhizal fungal communities differs among the roots, spores and extraradical mycelia associated with five Mediterranean plant species. Environ. Microbiol. 17:2882–2895. doi:10.1111/1462-2920.12810
66. Marleau J, Dalpé Y, St-Arnaud M, Hijri M (2011) Spore development and nuclear inheritance in arbuscular mycorrhizal fungi. BMC Evol. Biol. 11:1–11. doi:10.1186/1471-2148-11-51
67. Hempel S, Renker C, Buscot F (2007) Differences in the species composition of arbuscular mycorrhizal fungi in spore, root and soil communities in a grassland ecosystem. Environ. Microbiol. 9:1930–1938. doi:10.1111/j.1462-2920.2007.01309.x
68. Maherali H, Klironomos JN (2007) Influence of phylogeny on fungal community assembly and ecosystem functioning. Science 316:1746–1748
69. Ranelli LB, Hendricks WQ, Lynn JS, Kivlin SN, Rudgers JA (2015) Biotic and abiotic predictors of fungal colonization in grasses of the Colorado Rockies. Divers. Distrib. 21:962–976. doi:10.1111/ddi.12310
70. Casas C, Omacini M, Montecchia M, Correa O (2011) Soil microbial community responses to the fungal endophyte *Neotyphodium* in Italian ryegrass. Plant Soil 340:347–355. doi:10.1007/s11104-010-0607-8
71. Franzluebbers AJ (2006) Short-term responses of soil C and N fractions to tall fescue endophyte infection. Plant Soil 282:153–164. doi:10.1007/s11104-005-5447-6