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REGULAR ARTICLE

Soil microbial community responses to the fungal endophyte *Neotyphodium* in Italian ryegrass

Cecilia Casas · Marina Omacini · Marcela Susana Montecchia · Olga Susana Correa

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Abstract Cool-season grasses commonly harbor fungal endophytes in their aerial tissues. However the effects of these symbionts on soil microbial communities have rarely been investigated. Our objective was to explore microbial community responses in soils conditioned by plants of the annual grass *Lolium multiflorum* with contrasting levels of infection with the endophyte *Neotyphodium occultans*. At the end of the host growing season, we estimated the functional capacity of soil microbial communities (via catabolic response profiles), the contribution of fungi and bacteria to soil activity (via selective inhibition with antibiotics), and the structure of both microbial communities by molecular analyses. Soil conditioning by highly infected plants affected soil catabolic profiles and tended to increase soil fungal activity. We detected a shift in bacterial community structure while no changes were observed for fungi. Soil responses became evident even without changes in host plant biomass or soil organic carbon or total nitrogen content, suggesting that the endophyte modified host rhizodepositions during the conditioning phase. Our results have implications for the understanding of the reciprocal interactions between above and belowground communities, suggesting that plant-soil feedbacks can be mediated by this symbiosis.

Keywords Above and below ground interactions · Aerial symbiosis · *Neotyphodium* endophytes · *Lolium multiflorum* · Soil fungi and bacteria

Abbreviations

+E	*Lolium multiflorum* population highly infected with the endophyte *Neotyphodium occultans*
−E	*Lolium multiflorum* population lowly infected with the endophyte *Neotyphodium occultans*
CP	catabolic response profile
SI	selective inhibition
DGGE	denaturing gradient gel electrophoresis
RFLP	restriction fragment length polymorphism

Introduction

Biotic interactions between living plants and aboveground organisms can influence below-ground

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C. Casas (✉) · M. Omacini
IFEVA-CONICET, Cátedra de Ecología, Facultad de Agronomía, Universidad de Buenos Aires,
Av. San Martín 4453,
C1417DSE Ciudad Autónoma de Buenos Aires, Argentina
e-mail: ccasas@agro.uba.ar

M. S. Montecchia · O. S. Correa
INBA-CONICET, Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires,
Av. San Martín 4453,
C1417DSE Ciudad Autónoma de Buenos Aires, Argentina

communities with important consequences on ecosystem functioning (De Deyn and van der Putten 2005; van der Heijden et al. 2008). Concepts and theories in this field have been almost exclusively based on plant responses to above ground herbivores (Wardle and Giller 1996). Foliar herbivory reduces the biomass entering the soil as dead organic matter (litter) and induces plant physiological responses, which determine the quantity and quality of root exudates and leachates (Bardgett et al. 1998). All these organic inputs to soil may influence the activity and structure of soil microbial community and, in turn, plant growth, generating feedback loops (Wardle et al. 2004). The current challenge is to include other biotic interactions that can also alter below and aboveground communities through the same pathways (van der Putten et al. 2009).

Asexual fungi from the genus *Neotyphodium* (commonly known as endophytes) grow within the aerial tissues of many cool-season grasses, causing a systemic and asymptomatic infection which is transmitted vertically through the host plant seeds (Clay and Schardl 2002). By living within the grass, the endophyte obtains nutrients, protection and a mode of propagation while inducing a range of physiological, biochemical and morphological responses in the host plant (Bush et al. 1997). For example, certain endophyte infected plants produce secondary compounds toxic for invertebrate or vertebrate herbivores (mainly alkaloids and phenolic compounds) that are responsible for cascading effects on multiple trophic levels (Omacini et al. 2001; Rudgers and Clay 2007). Likewise, endophyte presence can influence the structure and dynamics of communities and ecosystems. Those effects have been generally associated with the infected host's competitive ability and its resistance to herbivory (i.e. Clay 1993). However, recent studies have shown that changes in the biological properties of soils mediated by the presence of endophyte-infected plants can alter establishment and growth of other plant species (Rudgers et al. 2007; Rudgers and Orr 2009).

Soil microbial community responses to the presence of the plant-endophyte symbiosis are essential to understanding the ecosystem functions. Specific experiments which have explored the combined response of soil microbes and invertebrate species to fungal endophytes are scarce, and their results are inconsistent (Jenkins et al. 2006; Lemons et al. 2005; Van Hecke et al. 2005). However, previous studies suggest that this symbiosis may reduce the rate of key soil ecosystem processes (e.g. decomposition and nutrient mineralization) regulated by soil communities (Omacini et al. 2004). For example, endophyte presence can modify litter decomposition by changing the quality of the litter produced by infected plants and by altering micro-environmental conditions (Lemons et al. 2005; Omacini et al. 2004). Moreover, previous studies indicated that the endophyte may reduce colonization by arbuscular mycorrhizal fungi either in the host plant (Mack and Rudgers 2008; Omacini et al. 2006) or in the plants growing in next generation after the death of host plants (Antunes et al. 2008).

The objective of this study was to explore the microbial community function and structure in soils conditioned by plants of the annual grass *Lolium multiflorum* (Italian ryegrass) naturally infected with the endophyte *Neotyphodium occultans*. This symbiosis constitutes model system to explore the impact of aboveground fungal endophytes on an annual host (Omacini et al. 2005), as most previous studies have focused primarily on perennial host plants (Saikkonen et al. 2006). In experimental microcosms, we grown plants with contrasting endophyte infection levels, and at the end of the growing season, we estimated (i) the soil function by measuring the respiration responses to different carbon substrates (catabolic profiles [CP] Degens and Harris 1997), (ii) the contribution of fungi and bacteria to the active soil microbial community by selective inhibition with antibiotics (SI, Susyan et al. 2005) and (iii) the community structure of both microbial groups. Additionally, we measured plant biomass, soil moisture, soil organic carbon and total nitrogen content considering that these variables may respond to endophyte presence (Franzluebbers and Stuedemann 2005) and thus, may influence soil community function (Zak et al. 2003).

Materials and methods

Experimental design

From March to December 2005 soils were conditioned by Italian ryegrass plants with high or low levels of endophyte infection (+E and −E plants) to obtain +E or −E soils at the end of the growing

season. The experiment, in a complete random design, included twenty-four pots (3 L, 20 cm diameter) sown with three +E or −E plants under outdoor conditions (experimental garden at Buenos Aires University, 34° 36′ S, 58° 26′ W). Mean temperature was 15°C during the cold season (March–September) and 20.3°C during the warm season (October–December), while precipitation totaled 716 and 138 mm for both seasons, respectively.

To obtain seeds with contrasting endophyte infection levels, 1 year before the experiment, we harvested Italian ryegrass seeds with high levels of endophyte infection (>90%) from a pampean grassland dominated by this symbiosis. Half of these seeds were treated with the fungicide Triadimenol (5 $mg g^{-1}$ seed) to kill the endophyte and to obtain uninfected seeds (details in Omacini et al. 2004). Untreated and treated seeds were sown in outdoor monocultures (under the same environmental conditions) and the seeds obtained from these plants were used to soil conditioning. Endophyte infection levels of these seeds were 90% and 2% (considered as +E and −E seeds, respectively). At the end of the plant's growing season, 30 seeds produced by the plants in the experimental pots were harvested and stained to verify endophyte infection level in the harvested seeds (Bacon and White 1994). Above and below ground biomass was also estimated by harvesting and oven drying (80°C, 3 days) tillers and roots of each pot (both expressed as g pot^{-1}). Three plants per pot were enough to find the plots plenty of roots at sampling.

All pots were filled with Natraquoll soil (4 mm sieved) mixed with sterilized sand (1:1 (v/v)). The soil used (0–10 cm deep) was taken from a grassland covered by humid mesophytic meadows in the Flooding Pampa (36° 30′ S, 58° 30′ W) (Perelman et al. 2001). In these grasslands, Italian ryegrass is commonly present but at low cover (less than 10%) and is naturally infected with *N. occultans* (Gundel et al. 2009). Soil parameters, expressed as soil dry weight (sdw), were: soil organic carbon (24 $g kg^{-1}$), total nitrogen content (2.2 $g kg^{-1}$), phosphorous (5.5 ppm), Na^{+}(0.64 $cmol_c\ kg^{-1}$), electrical conductivity (1.54 mmhos cm^{-1}) and pH (6.7). At the end of the plant biological cycle in December soil cores (6 cm diameter, 2–8 cm deep) were taken from each pot. These soil cores were immediately sent to the laboratory in order to avoid traditional conservation methods that can alter soil communities (Ross et al. 1980). Soil was sieved (4 mm) to remove large roots and animals. Sub-samples from the sieved soil were used to all the analyses. Soil gravimetric moisture (%) was determined by drying soil at 80°C during 3 days, total nitrogen content by the micro Kjeldahl method and soil organic carbon by the Walkley Black method.

Soil function was estimated using the catabolic response profile technique (hereinafter, CP) which is based on the fact that different species have different capacities to metabolize a range of substrates and so the response of whole soil communities to multiple substrates is an indicator of the potential functional capability of the organisms present (Degens and Harris 1997; Schipper et al. 2001; Stevenson et al. 2004). Short-term respiration responses of +E and −E soils were measured after the addition of 11 different carbon compounds: glutamic acid, malic acid, oxalic acid, asparagine, lysine, starch, cellulose, galactose, glucose, +E litter and −E litter (naturally dried and finely cutted). The two types of litter were obtained from plants with contrasting infection levels cultivated during the previous growing season under the same environmental conditions (as explained for seed production). The carbon compounds used accounted for a range of quality (carbohydrates, carboxylic acids, aminoacids, litter) and complexity (i.e. glucose < starch < celullose). Omacini et al. (2004) observed that soil conditioning by +E reduced the rate of litter decomposition of other non endophytic grass (*Bromus unioloides*). Thus, by including litter from both +E and −E plants we evaluated the capability of +E or −E soils in using litter from equal or different endophytic level.

Each substrate was mixed with talcum powder (1:3) and added to soil samples (18 g fw) in concentrations as indicated by Degens and Harris (1997). Talcum has been proven effective in the application of substrates in aqueous solutions and adequate to determine basal respiration (Anderson and Domsch 1978; Susyan et al. 2005). After one stabilization hour, one alkali trap (10 ml, 0.05 M NaOH) was placed inside each bottle which was immediately sealed. Bottles with soil were incubated for 5 h at 22°C under dark conditions. CO_2 evolved from each soil sample was determined by titrating the alkali traps (0.05 M HCl) and expressed as respiration rates (μg $CO_2\ g^{-1}$ sdw h^{-1}).

The contribution of fungi and bacteria to the active soil microbial community was assessed using anti-

biotics to distinguish fungal and bacterial substrate-induced respiration (hereinafter, SI) (Susyan et al. 2005). Four soil sub-samples (2.5 g of soil fresh weight (sfw)) from each pot were enriched with glucose (4 $mg\,g^{-1}$). After 1 h of stabilization, the four sub-samples were amended with streptomycin (10 $mg\,g^{-1}$ sfw) for bacterial inhibition, cycloheximide (25 $mg\,g^{-1}$ sfw) for fungal inhibition, both antibiotics, or without antibiotics (control). Glucose and antibiotics were mixed with talcum powder (1:3) as explained for CP method. Preliminary experiments were performed to determine the concentrations of antibiotics that provide for the maximum soil microbial inhibition (data not shown). Hermetic sealed vials were incubated for 5 h at 22ºC, under dark conditions. Headspace CO_2 concentration was measured by gas chromatography (GC 5890 Series II Hewlett Packard) and expressed as respiration rates ($\mu g\ CO_2\ g^{-1}$ sdw h^{-1}).

The Inhibitor Additivity Ratio (IAR) for the selected antibiotics, streptomycin and cycloheximide was calculated using the formula $IAR = [(A - B) + (A - C)]/(A - D)$, where A is the respiration rate with glucose, B is the respiration rate with glucose and cycloheximide, C is the respiration rate with glucose and streptomycin, and D is the respiration rate with glucose, streptomycin, and cycloheximide. The contribution of fungi and bacteria to soil respiration (FR and BR, respectively) was calculated using the formulas $FR = (A - B)/(A - D) \times 100\%$, $BR = (A - C)/(A - D) \times 100\%$ where A, B, C and D has the same definitions as in the previous formula. Technical and economic constraints determined that, in this case, only six replicates from each +E and −E treatment were analyzed.

The structure of soil microbial communities was assessed by two comparable ribosomal-based fingerprinting methods (Smalla et al. 2007). We applied a denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments to analyze bacterial community and restriction fragment length polymorphism (RFLP) profiling of fungal 18S rRNA genes to analyze the fungal community. Total community DNA from +E and −E treatments were extracted from 0.5 g of soil samples (five independent replicates per treatment) by UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.) according to the manufacturer's protocol. PCR and DGGE analysis of 16S rDNA were performed as described by Correa et al. (2009). PCR and RFLP analysis of 18S rRNA genes were performed following the protocol described by Schwarzenbach et al. (2007) using DNA of *Rhizoctonia* sp. as positive control for PCR reactions.

Data analysis

For each plant and soil variable (and below ground biomass, soil gravimetric moisture, soil total nitrogen, soil organic carbon and soil basal respiration), Welch *t*-test was used to test for effects of endophyte treatments. This test provides for adequate compensation when unequal variances are found (Ruxton 2006).

The catabolic responses of +E and −E soils were analyzed by estimating the 95% confidence interval for the difference between each substrate and the corresponding basal respiration (net catabolic response of soil). Each confidence interval was estimated by bootstrap with 4000 replacing re-samples (Wood 2005) using Infostat software (InfoStat 2008). We tested whether net catabolic response of soil differed significantly from zero. If 95% of these bootstrap estimates fell either above or below zero, the estimates were considered significantly different from zero.

The IAR, FR and BR from +E or −E soils were compared by Welch *t*-test. The IAR values obtained were similar and near 1 (+E 1.14 and −E 1.09 respectively, Welch *t*-test, $t_{9.924}=0.817$, $p=0.4$) indicating an equal antibiotic effectiveness in both treatments and that streptomycin and cycloheximide showed neither an additive nor antagonistic effect (Susyan et al. 2005). We performed Pearson product-moment correlations between above-ground biomass, below-ground biomass, soil organic carbon, total nitrogen contents or soil moisture and the activity of fungi or bacteria (R Development Core Team 2007).

Similarities of bacterial community DGGE profiles and fungal community RFLP profiles between samples were estimated by cluster analysis. Normalizations and analyses of DGGE and RFLP gel patterns were done with the software GelCompare II v.3.0 (Applied Maths NV). The normalized banding patterns were used to generate dendrograms by calculating the Pearson's product moment correlation coefficient and by unweighted pair group method with arithmetic averages (UPGMA) clustering (Sneath and Sokal 1973).

Results

At the end of the plant growing season, there were not significant differences ($p>0.05$) in the above- and belowground biomass of *Lolium multiflorum* plants with high or low endophyte infection level (Table 1). Moreover, there were not significant differences in soil moisture, soil nitrogen, or soil organic carbon between soil conditioned by those plants (+E and −E soils, Table 1). Neither plant nor soil variables were clearly associated with the contribution of bacteria or fungi to soil respiration (Table 2). These results indicate that endophyte effects, if any, were not mediated through these variables.

Soil microbial community responses to carbon compounds depended on the conditioning treatment (Fig. 1). Basal respiration did not differ between +E and −E soils ($t_{16.461}=1.1723$, $p=0.257$). However, the following, endophyte-dependent, changes in the pattern of substrate utilization were observed: carboxylic acids and cellulose reduced soil respiration in −E soils whereas glucose, starch and +E litter stimulated respiration in +E soils. In contrast, the addition of amino acids did not affect the metabolic activity of either of the soils. (Fig. 1).

Soil bacterial activity was 5 and 7.5 times higher than soil fungi activity in +E and −E soils, respectively (+E: $t_{8.901}=20.03$, $p<0.0001$; −E: $t_{8.543}=16.59$, $p<0.0001$). The endophyte infection level of the plants grown in the soils altered fungal activity, increasing +E levels increased fungal activity (Fig. 3, $t_{9.448}=1.9488$, $p=0.08$) but did not affect bacterial activity (Fig. 2).

Molecular analyses revealed that soil conditioning induced a slight shift in the structure of soil microbial communities. DGGE analysis of PCR-amplified 16S rRNA gene fragments clearly separated the +E and −E soils (Fig. 3a). The DGGE banding patterns of each treatment clustered separately but shared at least 85% similarity. This results suggest that endophyte infection level induced a small, but detectable, shift in the genetic structure of bacterial communities. In contrast, +E and −E soils clustered together in the UPGMA analysis of soil fungal communities (Fig. 3b) revealing that we were not able to detect a shift in the structure of this soil microbial group in response to conditioning by plants with different endophyte infection.

Discussion

Our results show that the presence of the fungal endophyte *N. occultans* in *L. multiflorum* plants can alter the function and structure of soil microbial communities. Endophyte negative effects on soil biota activity have been generally associated to the above-ground litter inputs of the host plants (Antunes et al. 2008; Lemons et al. 2005; Omacini et al. 2004). Instead, this study demonstrates that 7 months, one growing season for an annual host, was enough to detect significant changes in the soil functional capacity (by using catabolic response profiles) and perceptible differences in the activity of soil fungi (by using selective inhibition with antibiotics) as well as in the structure of bacterial communities in relation to

Table 1 Mean values for soil parameters and above and below ground biomass of *Lolium multiflorum* infected or uninfected with *Neotyphodium occultans* (+E and −E, respectively) at the end of the growing season ($n=12$)

	Treatment			
	+E	−E	*t*	*p*-value
Lolium multiflorum biomass				
Above ground (g dw. pot^{-1})	2.58 [a]	2.77 [a]	0.86	0.39
Below ground (g dw. pot^{-1})	4.95 [a]	5.10 [a]	0.22	0.82
Soil parameters				
Soil moisture (%)	15.73 [a]	15.61 [a]	0.15	0.87
Organic carbon (g C kg^{-1} sdw)	11.66 [a]	11.52 [a]	0.19	0.84
Total nitrogen (g N kg^{-1} sdw)	1.01 [a]	1.06 [a]	0.61	0.54

Different letters indicates significant differences between treatments for each variable (Welch *t*-test, $p<0.05$)

Table 2 Pearson product-moment correlations between above ground biomass, below ground biomass, soil moisture, soil organic carbon or total nitrogen contents and the relative contribution of fungi or bacteria to the total soil respiration as estimated with selective inhibition (n=12)

	Fungal respiration (%)		Bacterial respiration (%)	
	r	p-value	r	p-value
Lolium multiflorum biomass				
Above ground (g pot^{-1})	−0.45	0.14	0.02	0.94
Below ground (g pot^{-1})	−0.15	0.64	0.04	0.90
Soil parameters				
Moisture (%)	−0.14	0.67	0.23	0.48
Organic carbon (gC kg^{-1})	0.02	0.95	0.16	0.63
Total nitrogen (gN kg^{-1})	−0.18	0.58	0.24	0.45

endophyte infection level. Our analyses proved to be effective for studying the belowground consequences of an endophyte naturally occurring in several exotic invasive grasses, even though we can only suggest the potential mechanisms involved in the soil responses (Clay and Schardl 2002).

Endophyte effects on soil activity became evident even without changes in host plant biomass, soil organic carbon or total nitrogen content in soil. Despite this experiment was not designed to identify the specific mechanisms, other studies suggest that differential root exudation between +E and -E modified the quantity/quality of host plant rhizodepositions (i.e. exudates) (Malinowski et al. 1998; Van Hecke et al. 2005). We did not measure the rhizodepositions of plants with different infection levels because we were particularly interested in evaluating effects on soil microbial communities under natural conditions where serious methodological problems prevent accurate measurements. Meanwhile, as far as we are aware, no one to date has done these measurements in soils conditioned by *L. multiflorum* plants. In culture media, Vila Aiub et al. (2003) detected an increment in root proton extrusion mediated by *N. occultans* presence in this grass. Previous works have also reported changes in the rhizosphere chemistry and enzymatic activity mediated by endophyte presence in perennial host grasses (Van Hecke et al. 2005; Malinowski et al. 1998). In particular, Malinowski et al. (1998) found an increase Fe^{3+} reducing activity induced by highly infected *L. arundinacea* plants. These results and our data support the hypothesis that endophyte infected plants may decrease the rhizosphere pH and thereby gener-

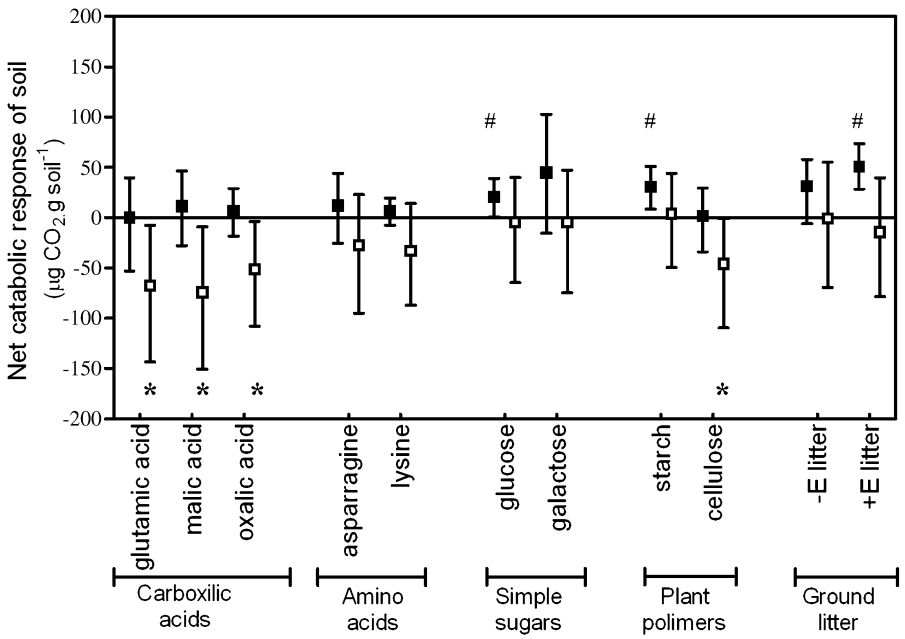

Fig. 1 Catabolic profiles for soil conditioned by *Lolium multiflorum* plants with high or low levels of endophyte infection (+E black or −E white, respectively). Net catabolic responses were calculated as the difference between the respiration rates after adding different substrates and the basal respiration. Squares represent the mean ± 95% CI; significant differences of each of the squares from zero are indicated with # for +E and * for −E

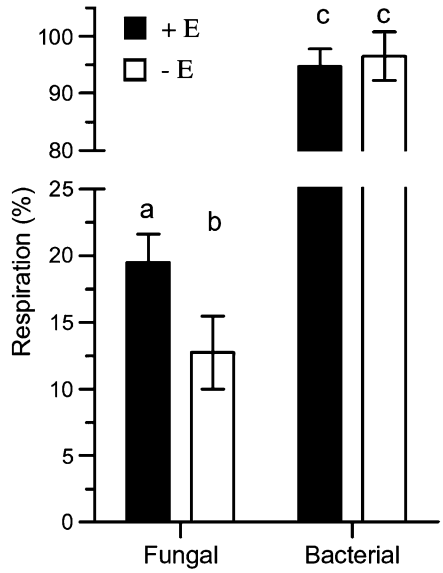

Fig. 2 The relative contribution of fungi and bacteria to the total soil microbial respiration. The bars show the mean ± standard error from soils previously conditioned by *Lolium multiflorum* with high level of infection (+E) or low level of infection (−E) with *Neotyphodium occultans* endophyte (n=6). Significant differences between means are denoted with different letters. Welch t-test (p-value <0.1)

ating an environment more favorable for fungi than bacteria (Rousk et al. 2009).

Endophyte induced changes in the soil capacity to metabolize a range of substrates support the hypothesis that the grass-endophyte symbiosis may alter ecosystem function in different ways (Omacini et al. 2004). Under our experimental conditions, without a litter layer, we detected that soil metabolic capacity increased with almost all the compounds in soils conditioned by highly infected *L. multiflorum* plants. Because of functional redundancy in soil microbial biota, it remains unclear to what extent the metabolic profile of each treatment is related to changes in either bacteria or fungi soil groups (Rousk et al. 2009). Nevertheless, we can explain certain patterns considering that bacterial activity is generally associated to the use of the simplest organic compounds (i.e. glucose) as well as fungal activity is linked to the use of the most recalcitrant compounds (i.e. lignin) (Horwath 2007).Thus, the subtle difference between soils in the response to glucose can be related to the lack of an endophyte significant effect on the activity of soil bacteria, the group responsible for nearly 80% of soil microbial respiration. Meanwhile, the difficulties observed to use cellulose and litter in soils conditioned by −E plants is coherent with a downward trend for fungal activity in those soils. However, we cannot discard that these soil responses and the low capacity to metabolize carboxylic acids can be associated to the disappearance of bacteria responsible of that metabolic route, as indicated by the perceptible difference between soils in the structure of bacterial communities.

It is important to remark that our approach showed that fungal activity tended to increase in soils conditioned by highly infected plants without a shift in the structure of this soil microbial community. According with our molecular analyses, the endophyte was only responsible for slight changes on the structure of the bacterial community. Within the context of this short-term experiment, the differences in the life cycle of both microbial groups, may

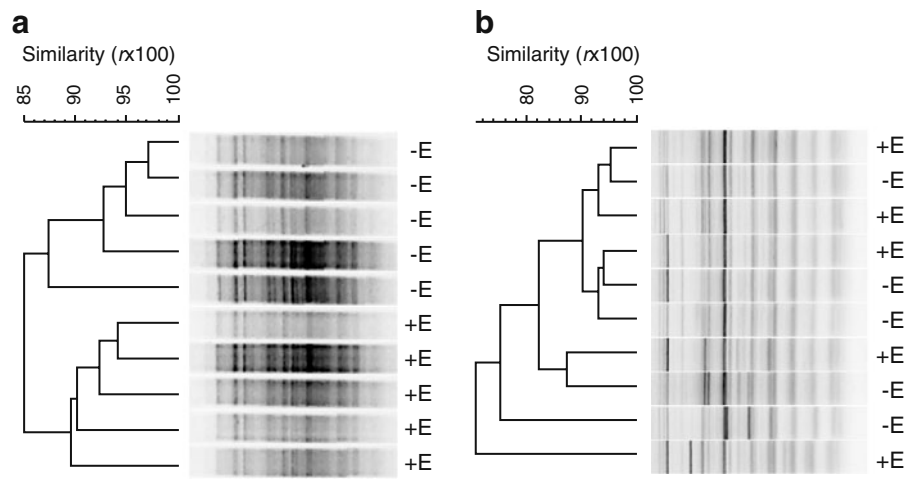

Fig. 3 Cluster analysis (Pearson/UPGMA) of ribosomal-based fingerprints from soil microbial communities previously conditioned by *Lolium multiflorum* plants with high level of infection (+E) or low level of infection (−E) with *Neotyphodium occultans* endophyte (n=5). Dendrograms of DGGE profiles for the bacterial (**a**), and RFLP profiles for the fungal communities (**b**)

account for this results (i.e. bacteria may change their community structure faster than fungi). In this sense, the effect of endophyte on soil fungi may be evident on a longer period. In addition, we have to consider that DGGE and RFLP methods are exploratory and semi-quantitative and, therefore, minor differences in soil microbial communities could not be detected. Understanding endophyte impact on ecosystem function requires studying the long-term implications of such belowground effects or the consequences when fungi do not represent a minimal percentage of soil microbial activity.

In summary, our study was aimed at measuring the effects of endophyte presence in host aerial tissues on soil microbial community structure and function. The generality of our conclusions is limited by our experimental design; however, our results clearly suggest that the endophyte occurring in annual invasive grass may cause changes in the soil ecosystem that have not been studied before. Different capacities to metabolize a range of substrates were found in soil microbial communities conditioned by *L. multiflorum* plants with contrasting levels of endophyte infection. Although historically overlooked, minor changes on soil microbial communities, may propagate on complex interaction webs affecting ecosystem function and dynamics (Rudgers and Clay 2007). Our results have implications for the understanding of the reciprocal interactions between above and belowground community subsystems, and suggest that plant-soil feedbacks can be affected by this symbiosis.

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