Mycol Progress (2009) 8:75–81
DOI 10.1007/s11557-008-0579-8

ORIGINAL ARTICLE

Positive association between mycorrhiza and foliar endophytes in *Poa bonariensis*, a native grass

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Received: 2 September 2008 / Revised: 18 November 2008 / Accepted: 2 December 2008 / Published online: 20 December 2008

Abstract The interaction between mycorrhiza and leaf endophytes (*Neotyphodium* sp.) was studied in three *Poa bonariensis* populations, a native grass, differing significantly in endophyte infection. The association between endophytes and mycorrhizal fungi colonisation was assessed by analysing plant roots collected from the field. We found that roots from endophyte-infected populations showed a significantly higher frequency of colonisation by mycorrhizal fungi and that soil parameters were not related to endophyte infection or mycorrhiza colonization. In addition, we did not observe significant differences in the number of AM propagules in soils of the three populations sites. We also report the simultaneous development of *Paris*-type and *Arum*-type mycorrhiza morphology within the same root systems of *P. bonariensis*. The co-occurrence of both colonisation types in one and the same root system found in the three populations, which differed in *Neotyphodium* infection, suggests that foliar endophytes do not determine AM morphology. The percentage of root length colonised by different types of fungal structures (coils, arbuscules, longitudinal hyphae and vesicles) showed significant and positive differences in arbuscular frequency associated with endophyte infection, whereas the much smaller amounts of vesicles and hyphal coils did not differ significantly.

Keywords Arbuscular mycorrhiza · *Arum*-type · Native grass · *Neotyphodium* · *Paris*-type · Plant-fungus interactions

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Introduction

Symbiosis between fungi and plants is a widespread phenomenon in nature (Kogel et al. 2006) and most organisms frequently connect in more than one interaction simultaneously (Richardson et al. 2000; Stachowicz 2001). Plants are commonly associated with mycorrhizal fungi, endophytic fungi, and nitrogen-fixing bacteria, among others, interacting with them in a mutualistic way. Most research has focused on pair-wise mutualisms, and data are directly obtained from manipulated interactions overlooking the complex interactions that take place in nature (Rudgers and Clay 2005; Mack and Rudgers 2008).

Foliar endophytes and arbuscular mycorrhizal fungi (AMF) are plant-fungal associations often considered mutualistic, as they can provide benefits to the host (Smith and Read 1997; Schardl et al. 2004). These two fungal symbionts are commonly hosted by grasses; however, studies on their potential interactions are scarce.

Endophytes of the genus *Neotyphodium* (Morgan-Jones and Gams) are obligate seed-borne fungi that commonly form intercellular infections in leaves, culms and inflorescences in many cool-season grasses in the subfamily Pooideae (Schardl et al. 1997). Interactions between plants and endophytic fungi can span the range from mutualistic to parasitic, depending, for example, on environmental conditions (Saikkonen et al. 2004; Müller and Krauss 2005;

Novas et al. 2007). These endophytes can provide grass with resistance to herbivory (Clay 1996), pathogen attack (Clay et al. 1989; Gwinn and Galvin 1992), drought stress (Elmi and West 1995), and increased tillering, reproduction and plant growth (Novas et al. 2003; Iannone and Cabral 2006).

Arbuscular mycorrhizal fungi associate with the roots of most of angiosperms (Smith and Read 1997, Brundrett 2002). The main benefit attributed to mycorrhiza is increased nutrient uptake from the soil, particularly of phosphorus and nitrogen (Smith and Read 1997). Other benefits may include enhancements of water uptake (Marulanda et al. 2003), photosynthetic rate (Black et al. 2000), and carbohydrate metabolism (Smith and Read 1997). Two main morphological types of AM were described by Gallaud (1905): the *Arum*-type, which is defined by intercellular hyphal growth in the root cortex, and the *Paris*-type, which is characterised by the cell-to-cell growth of intracellular hyphal coils. It is discussed whether the AM morphological type depends on the plant species (Brundrett and Kendrick 1990) or on the AM fungal genome (Abbott 1982; Cavagnaro et al. 2001).

In manipulated experiments, performed on the artificially selected agronomic grasses, *Lolium perenne*, *Lolium multiflorum* and *Lolium arundinaceum*, fungal endophytes have been reported to inhibit mycorrhiza (AM) colonization, (Chu-Chou et al. 1992; Guo et al. 1992; Müller 2003; Omacini et al. 2006; Liu et al. 2007; Mack and Rudgers 2008). However, the way in which endophyte infection and mycorrhizal colonization are correlated in natural grass-endophyte systems has been poorly studied.

An increasing number of papers indicate that the effects of the endophytes in manipulated experiments are dependent on the genotype of the endophyte and its host, nutrient level and experimental conditions (Cheplick et al. 2000; Ahlholm et al. 2002; Lehtonen et al. 2005). Moreover, the results of experiments conducted on agronomic grasses may fail to capture the breadth of variability inherent in wild grass-endophyte populations and communities and are unrepresentative of the relevant biological diversity (e.g. species and genetic diversity) and environmental variation to make appropriate generalizations about the importance of endophytes in wild grasses in nature (Saikkonen et al. 2006).

Field surveys and manipulated experiments indicated that roots of endophyte infected plants of *Bromus setifolius*, a native grass, are colonised more extensively by AMF than those of endophyte-free populations (Novas et al. 2005). Furthermore, Chen et al. (2007) have suggested that endophytes may not affect mycorrhizal colonisation directly.

Information about endophytes in wild growing plants is generally limited, and their effects within the greater plant-fungus system are therefore of considerable interest. Because of the contrasting results between agronomic grasses and wild native grasses regarding the relationship between endophyte and mycorrhizal colonisation, additional wild native grasses-endophyte-mycorrhyza systems should be studied.

The aim of this study was to examine the association between *Neotyphodium* endophytes and mycorrhiza fungi present in *Poa bonariensis* (Lam.) Kunth. To this end, we quantified mycorrhiza and studied the types of mycorrhiza morphology.

Materials and methods

Study system

Populations of *Poa bonariensis*, a native and perennial grass, found at Punta Indio, Buenos Aires province, Argentina, were selected for the present study. These populations represent an interesting natural model to investigate multitrophic associations because they grow very near to each other, showing significant differences in *Neotyphodium* colonisation among them.

Punta Indio is located at the "Parque Costero del Sur", which has been designated as a Biosphere Reserve by the UNESCO (http://www.unesco.org/mab/wnbrs.shtml). The Costero del Sur Biosphere Reserve is located in the Province of Buenos Aires on the southern coast of the Rio de la Plata estuary on the Atlantic coast (36° 00′ S, 57° 30′ W), Argentina. It is situated in a humid and swampy region that comprises pampas grasslands characterised by deep, fertile soils. It includes coastal areas, flooded and unflooded swamps, wetlands and dry forests. Punta Indio, particularly, is a region characterised by the presence of xerophytic forests on shelly ridges characterized by the presence of "Tala" (*Celtis tala* Planchon). Tala forests ("talares") occur in high, well-drained areas (Cabrera 1976) and the forests are considered as edaphic communities, conditioned by calcareous soil and subsoil (Cabrera 1949, 1976; Verboorst 1967). *P. bonariensis* populations are associated with "Tala" forests (*Celtis tala* Planchon) and mycorrhizal fungi such as *Acaulospora delicata* Walker, Pfeiffer & Bloss, *Scutellospora dipapillosa* (Walker & Koske) Walker & Sanders, *S. fulgida* Koske & Walker, *S. gilmorei* (Trappe & Gerd.) Walker & Sanders, *Glomus clarum* Nicolson & Schenck and *G. etunicatum* Becker & Gerdemann, have been previously observed in roots of plants associated with "talares" (Irrazabal et al. 2005).

The three populations selected for the present study were the only ones that exists in the area: population 1 (P1) 35° 16′ 55″ S 57° 11′ 06″ W; population 2 (P2) 35° 24′ 20″ S, 57° 11′ 06″ W and population 3 (P3) 35° 33′ 16″ S, 57° 13′ 32″ W. Populations P1 and P3, the most distant from each other, were approximately 24.7 km apart, while P1 and P2 were 8.7 km apart and P2 and P3 were 17.9 km apart.

Plant material

Poa bonariensis (Lam.) Kunth is a perennial species distributed in the northeast of Buenos Aires province, and Entre Rios province, Argentina, and also in Uruguay and southern Brazil. Plants are rhizomatous, 45-80-cm tall and dioecious. Leaf-sheaths are smooth and leaf-blades are flat or conduplicate; 15–30 cm long; 2–6 mm wide. The inflorescence is a panicle (Giussani 2000; Clayton et al. 2007). In natural grasslands it is eaten by cattle and considered as good forage (Cabrera 1970).

Twenty plants of *P. bonariensis* were randomly collected from each of the three selected natural populations. Shoots and roots were removed from each plant and separately stored in plastic bags for further laboratory analysis.

Seeds were collected from all the populations in order to carry out experimental assays in controlled conditions. However, germination rate was lower than 2% and seedlings did not survive. Even though, pre-treatments to induce germination were used, the germination rate was still lower than 2%. Therefore, we conducted a survey evaluating the association between endophyte infection and mycorrhiza colonization using plants collected from wild populations.

Endophyte infection

At least two culms of each plant were assessed for the presence of endophytic mycelium by examination of culm tissue using a light microscope. Endophytic mycelium was visualised by staining tissue scraped from within culms with aniline blue (0.1% aqueous) (Clark et al. 1983). Culms were identified as endophyte-infected if typical non-branching intercellular mycelium was evident among plant parenchyma tissues (White 1987). Differences in endophyte infection were analysed using the Chi-squared test.

Mycorrhizal colonisation

Mycorrhizal colonization was studied among the three populations and between E+ and E- plants within P2 (E+/E- population). In order to estimate the mycorrhizal colonisation level, the whole root system of 10 plants from each population was used and carefully washed with tap water to remove free soil, and then preserved in vials with FAA (10% formalin-5% acetic acid- 50% ethanol). The roots were stained in Trypan blue (Phillips and Hayman 1970) and 30 pieces per plant, each approximately 1 cm long, were randomly selected. The presence or absence of AMF (F%) and the extent of root cortex colonisation (M%) was recorded as described by Trouvelot et al. (1986) using the MYCOCALC programme (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

The morphological types of colonisation by AM fungi were also studied. Colonisation was assessed using the magnified intersections method (McGonigle et al. 1990) to obtain the percentage of root length colonised by each of various types of fungal structures: coils, arbuscules, longitudinal hyphae and vesicles.

Differences in mycorrhizal colonisation percentages among populations and differences between infected plants and non-infected plants within the E+/E- population (P2) were analysed by the Kruskal–Wallis test.

Soil parameters

To study a possible relationship between soil parameters and colonisation of both fungal symbionts, five soil samples of the upper horizon (10–30 cm) were taken from the three sites and these were mixed for each population. Samples were subjected to the following measurements,: pH in water 1:25, total C (Walkley-Black), total N (Kjeldahl, modified by Ritcher (1980)), C.E.C. (ammonium acetate 1N, pH 7), Ca++, Mg++, Na+ and K+ by the Laboratorio de Química Geológica y Edafológica-CONICET, Buenos Aires, Argentina.

Enumeration of AM fungal spores

Five rhizosphere samples of soil were collected at depths of 20–30 cm in each population site. Composite soil samples were mixed and kept at 5°C until used. The number of spores in three 5 g subsamples of each soil was determined using a modified wet sieving and decanting (Gerdemann and Nicolson 1963).

Five grams of soil from each population site were dispersed in 1 L water and decanted through a series of 500μm to 63μm sieves and this procedure was performed on three replicates. All intact spores were counted using a dissection microscope at x40 magnification. Sporocarps and spore clusters were considered as one unit. The differences in the spore number between populations were tested using the Kruskal–Wallis test.

Results

Endophyte infection

The endophytic incidence differed significantly between populations (χ^2=20; P<0.0001; df=2). Population P1 showed 100% of infection (= E+), P2 (= E+/−) 50% and P3 (= E−) 0%.

Mycorrhizal colonisation

Arbuscular mycorrhizal fungal structures were observed in roots of all examined populations. However, the extent of root colonisation varied significantly between populations (H= 10.97; P=0.0041). Roots of both endophyte-infected populations showed higher mycorrhizal colonisation (Fig. 1). Total

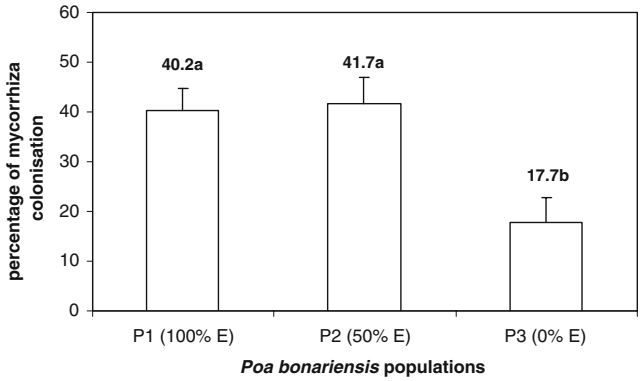

Fig. 1 Mycorrhizal fungi colonisation percentage between native populations of *Poa bonariensis* differing in *Neotyphodium* endophyte colonisation. Different letters above bars indicate significant differences in mycorrhiza colonisation between populations (Kruskal–Wallis test, p=0.05); 100% E=100% endophyte infected; 50% E=50% endophyte infected; 0% E = plants without endophytes

colonisation was 40.22% in P1, 41.69% in P2, and 17.66% in P3.

Comparisons of means indicated two groups of populations: one composed by P1 (E+) and P2 (E+/−), which fit together in one group showing the highest mycorrhizal colonisation, and another one formed by P3 alone.

Differences in root colonisation percentage between E+ (39.3±13. 2) and E- (28.5±7.59) plants within P2, E+/E- population, (H=1.35; P=0.2453) were not significant.

Morphological types of colonisation

Both types of AM morphology were observed in the three populations studied (Figs. 2-3), although the *Arum*-type was more frequently found than the *Paris*-type.

Some of the examined AM plants with both intercellular hyphae and intracellular spread of hyphae coils were categorised as intermediate type as described by Yamato (2004). However, in this intermediate type, a single infection gave rise to a body of infection that was consistent within itself as either *Arum*- or *Paris*-type.

Differences in colonisation by hyphal coils and vesicles were not significantly between populations, $H=0.18$, $P=0.91$ and $H=0.74$, $P=0.69$, respectively (Fig. 4), whereas differences in colonisation by arbuscules between populations were significant higher ($H=8.54$, $P=0.013$) for P1 and P2 (Fig. 4). Results in total hyphal colonisation measured following McGonigle et al. (1990) showed the same findings as those obtained following Trouvelot et al. (1986).

Soil parameters

We did not observe any relationship between endophyte infection nor mycorrhizal colonisation and soil parameters (Table 1).

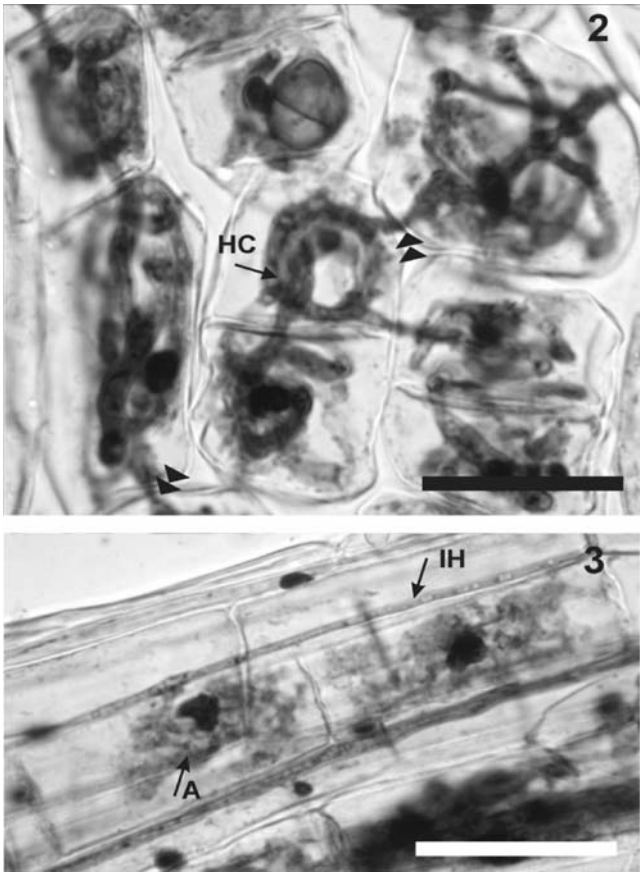

Fig. 2-3 Representative micrographs showing mycorrhizal association in *Poa bonariensis* (*HC* hyphal coil, *A* arbuscules, *IH* intercellular hyphal, *double arrowheads* cell to cell spread of colonisation). Both micrographs were taken from roots of the same plant, bar=50 µm. Figure 2 *Paris*-type mycorrhiza morphology, characterised by coils formed by hyphae penetrating adjacent cells. Hyphal spread from cell to cell is evident. Figure 3 *Arum*-type mycorrhiza morphology, characterised by intercellular hyphae between the cortical cells and intracellular arbuscules within them

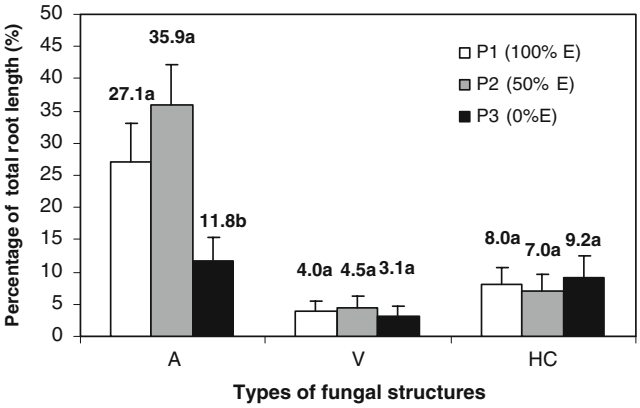

Fig. 4 Percentage of root length of *Poa bonariensis* native plants colonised by each of various types of fungal structures: arbuscules, hyphal coils and vesicles. *A* arbuscules, *V* vesicles, *HC* hyphal coils. Values are means ± SE for percentage of total root length. Different letters above bars, for the same structure, indicate significant differences between populations (Kruskal–Wallis test, p=0.05)

Table 1 Soil parameters in *Poa bonariensis* populations

Soil parameters	P1	P2	P3
pH	7.5	7.5	7.8
CE	0.66	0.56	0.65
C	23.74	13.86	14.22
C/N	13.11	11.36	9.61
N	1.81	1.22	1.48
P	1	12.8	0
CIC	9.6	10.9	17.8
Ca^{++}	22	17.3	19.1
Mg^{++}	0.3	0.3	0.2
Na^{+}	0.2	0.2	0.2
K^{+}	0.2	0.6	0.5

AM potential inoculum

Using the wet sieving technique, soil from P1, P2 and P3 was found to contain 13, 16 and 25 spores per 5 g soil, respectively .The Kruskal–Wallis test indicated that there are no significant pairwise differences among the means (H=2.388; P=0.3029). The morphotypes observed agree with species previously mentioned to be present in the studied area by other authors (Irrazabal et al. 2005).

Discussion

In the present study, we found a positive association between mycorrhizal fungi and *Neotyphodium* endophytes. Furthermore, we did not observe significant differences in the number of AM propagules among populations. These results support the hypothesis about a positive effect of *Neotyphodium* endophytes on mycorrhiza. It agrees with results from a previous study performed on *Bromus setifolius* (Novas et al. 2005) where *Neotyphodium* endophyte colonisation was positively correlated with AM colonisation in both field and experimental conditions.

Guo et al. (1992) have suggested that alkaloids produced by foliar endophytes (review: Powell and Petroski 1992) may be responsible for an inhibitory effect on AM fungi. Others propose a nutrient competition between both fungal symbionts or a systemic induction of resistance mechanisms of the host plant by the leaf endophyte, reducing mycorrhiza colonization (Müller 2003).

Alternatively, flavonoids have been suggested to stimulate mycorrhizae (Nair et al. 1991; Xie et al. 1998. This hypothesis is supported by the observation that endophyte-infected plants of *Poa ampla* produce this type of compounds (Ju et al. 1998). No specific phytochemical information is available to date for *Poa bonariensis*. In any case, the exact mechanisms that would explain the different mycorrhizal fungi colonisation rates observed between *Neotyphodium*-infected and free plants have not been identified yet (Liu et al. 2007).

Infected and non-infected plants within the same population (P2) showed similar AM colonisation percentages. This fact could be a consequence of below-ground links through mycorrhiza. It has been suggested that grassland plants may be connected to each other by hyphae of mycorrhizal fungi (Chiariello et al. 1982; Read et al. 1985). If E+ plants exude soil compounds that promote mycorrhiza development and colonization, neighbour E- plants could be more extensively infected. In addition, as mycorrhiza-colonised roots act as inoculum of mycorrhiza the higher colonisation of E+ plants could represent an increased inoculum in soil. Thus the mycorrhizal colonisation of E- plants could be enhanced when E+ and E- plants grow together.

A simultaneous development of separate and internally consistent infection units of *Paris*-type and *Arum*-type mycorrhiza was found within the same root systems of *P. bonariensis*. In previous work, most of the examined plant species belonging to the Poaceae family were categorised as intermediate (Yamato 2004). In the context of our results, we agree with Kubota et al. (2005), who have suggested that the morphology of arbuscular mycorrhiza is the result, at least in part, of the interplay between both the plant and the fungal species.

The co-occurrence of both colonization types in the three populations of *P. bonariensis*, differing in *Neotyphodium* infection, suggests that foliar endophytes do not determine the AM morphology.

The percentage of root length colonised by each of various types of fungal structures, coils, arbuscules, longitudinal hyphae and vesicles, was in part different in the three populations. We found significant positive differences between populations in arbuscular frequency. This is a remarkable result considering the predominant role of arbuscules in mycorrhiza physiology and should be taken into consideration in future studies. By contrast, the amount of vesicles and hyphal coils did not differ significantly between the populations. Further research is needed to demonstrate a causal relationship between amount of endophyte colonization and arbuscular development; presently only the correlation of higher occurrence of both with the particular grass populations can be stated.

It is noteworthy that all studies that found an antagonistic relationship between the fungal symbionts have been performed on agronomic grasses (Chu-Chou et al. 1992; Guo et al. 1992; Omacini et al. 2006; Liu et al. 2007), while those where a positive interaction was observed were performed on wild grasses. Thus additional surveys in natural populations of wild native grasses and manipulated experiments should be done in order to establish how the complex endophyte-grass-mycorrhiza interactions are affected in nature.

This research expands current knowledge to the association among endophytes and mycorrhiza and is an example of a study that takes a non-pair-wise view of mutualism. Our results support previous studies suggesting that this pattern could be widespread under field conditions and also provides data on endophytes in a native, wild grass.

Acknowledgements This research was supported by UBACyT X833, PIP 5311 and BID 17/28 OC-AR PICT 32251. PRHIDEB publication no. 173.

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