Different native arbuscular mycorrhizal fungi influence the coexistence of two plant species in a highly alkaline anthropogenic sediment

Rui S. Oliveira · Paula M. L. Castro · John C. Dodd · Miroslav Vosátka

Abstract Different species of arbuscular mycorrhizal fungi (AMF) can produce different amounts of extraradical mycelium (ERM) with differing architectures. They also have different efficiencies in gathering phosphate from the soil. These differences in phosphate uptake and ERM length or architecture may contribute to differential growth responses of plants and this may be an important contributor to plant species coexistence. The effects of the development of the ERM of AMF on the coexistence of two co-occurring plant species were investigated in root-free hyphal chambers in a rhizobox experimental unit. The dominant shrub (*Salix atrocinerea* Brot.) and herbaceous (*Conyza bilbaoana* J. Rémy) plant species found in a highly alkaline anthropogenic sediment were studied in symbiosis with four native AMF species (*Glomus intraradices* BEG163, *Glomus mosseae* BEG198, *Glomus geosporum* BEG199 and *Glomus claroideum* BEG210) that were the most abundant members of the AMF community found in the sediment. Different AMF species did not influence total plant productivity (sum of the biomass of *C. bilbaoana* and *S. atrocinerea*), but had a great impact on the individual biomass of each plant species. The AMF species with greater extracted ERM lengths (*G. mosseae* BEG198, *G. claroideum* BEG210 and the four mixed AMF) preferentially benefited the plant species with a high mycorrhizal dependency (*C. bilbaoana*), while the AMF species with the smallest ERM length (*G. geosporum* BEG199) benefited the plant species with a low mycorrhizal dependency (*S. atrocinerea*). Seed production of *C. bilbaoana* was only observed in plants inoculated with *G. mosseae* BEG198, *G. claroideum* BEG210 or the mixture of the four AMF. Our results show that AMF play an important role in the reproduction of *C. bilbaoana* coexisting with *S. atrocinerea* in the alkaline sediment and have the potential to stimulate or completely inhibit seed production. The community composition of native AMF and the length of the mycelium they produce spreading from roots into the surrounding soil can be determinant of the coexistence of naturally co-occurring plant species.

Keywords Arbuscular mycorrhizal fungi · Community composition · Extraradical mycelium · Plant diversity · Plant reproduction · Specificity

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Introduction

How plant species manage to coexist is an important question in community ecology (Barot and Gignoux 2004; Silvertown 2004). Arbuscular mycorrhizal fungi (AMF), from the phylum Glomeromycota, are a group of soil organisms that form symbiotic associations with roots of the majority of terrestrial plant species (Smith and Read 1997). The contribution of AMF to plant species coexistence has often been neglected, but there has been recent evidence that AMF can mediate plant species coexistence (Hart et al. 2003; van der Heijden et al. 2003).

The ability of many plant species to coexist determines plant biodiversity. Plant community diversity (Francis and Read 1994; Grime et al. 1987; Klironomos et al. 2000; van der Heijden et al. 1998b) and the balance of competition between plant species (Hartnett and Wilson 2002; Kytöviita et al. 2003; Marler et al. 1999) are strongly influenced by AMF.

The extraradical mycelium (ERM) of AMF is a key component of the arbuscular mycorrhizal symbiosis linking colonised roots with the soil matrix (Dodd 1994). One of the most important roles of the ERM in plant growth is the uptake and translocation of phosphate from soil to the roots (Dodd et al. 2000). AMF species can produce different amounts of ERM and can have different efficiencies in gathering phosphate (Helgason et al. 2002; Jakobsen et al. 1992; Jansa et al. 2005; Munkvold et al. 2004). Moreover, AMF species can differ with respect to phosphate metabolism in the ERM (Boddington and Dodd 1999) and can show spatial differences in acquiring soil phosphate (Smith et al. 2000). These differences in phosphate uptake and ERM length or architecture may contribute to differential growth responses of plants (Helgason et al. 2002; Jakobsen et al. 1992; Munkvold et al. 2004) and this may be an important contributor to plant species coexistence (van der Heijden et al. 2003). Furthermore, the differential growth response of plants to different AMF can be great when locally adapted plants and fungi are used (Klironomos 2003). It is, therefore, important to understand the effect of different native AMF species with different ERM lengths and architectures on plant species coexistence.

Promoting plant coexistence is necessary in phytorestoration programs aiming to maintain plant diversity. Plant reproduction on sites targeted for phytorestoration is essential for subsequent survival and spread of the plant species in the field. AMF have been shown to positively influence plant reproduction (Dodd et al. 2002; Koide 2000b). AMF colonisation was also associated with an increase in phosphate uptake when AMF colonisation increased plant reproduction (Koide 2000b). Plant reproduction may be more responsive to colonisation by AMF in habitats where there is a great phosphate deficit (e.g. degraded and anthropogenic sites) (Dodd et al. 2002; Koide and Dickie 2002). Sanders and Koide (1994) demonstrated that, in a plant community, the presence of AMF decreased reproduction of a non-mycotrophic plant species and increased the reproduction of a strongly mycotrophic plant species. Since different AMF species can take up and transfer phosphate to plants with various efficacies, then it is possible that AMF also have a variety of influences on plant reproduction.

Although AMF are present in most terrestrial biomes (Brundrett 1991), it is unlikely that their influence on plant growth and coexistence is identical in all ecosystems. Studies on the influence of AMF in plant coexistence and plant community structure in anthropogenic habitats are scarce (Malcová et al. 1999). Of special interest are sites targeted for phytorestoration schemes, where plant species with different mycorrhizal dependencies are being considered, since it has been suggested by van der Heijden (2002) that a positive relationship exists between the mycorrhizal dependency of a plant species and the degree to which it responds differently to different AMF species. Oliveira et al. (2001, 2005b) reported the occurrence of arbuscular mycorrhizas on plants growing in a highly alkaline anthropogenic sediment in Northern Portugal. The site had a depauperate vegetation cover of limited diversity and the arbuscular mycorrhizal plants accounted for a large percentage of total plant abundance. Phytorestoration practices are being adopted to rehabilitate the site. The biological significance of AMF for plant coexistence may be pronounced in very harsh environments, such as highly alkaline anthropogenic sediments,

where low phosphate availability and high pH and salinity are limiting factors for plant survival and growth.

The aim of the present work was to determine the influence of different native AMF species, alone and together, on the coexistence of two naturally co-occurring plant species in a highly alkaline anthropogenic sediment. *Conyza bilbaoana* (Asteraceae) was the dominant herbaceous plant species on the study site and had a high mycorrhizal dependency and *Salix atrocinerea* (Salicaceae) was the dominant shrub and had a low mycorrhizal dependency (Oliveira et al. 2001, 2005b; personal communication). These plant species are often found growing in the vicinity of each other on the site. *Conyza bilbaoana* and *S. atrocinerea* were inoculated in microcosms containing the alkaline sediment with one of four different native AMF species or a mixture of all four. Plant and fungal development and the reproduction of *C. bilbaoana* were evaluated.

Materials and methods

Anthropogenic sediment, plant and fungal material

Both the plants and AMF studied in this work occur in the same highly alkaline anthropogenic sediment in Northern Portugal (40 46′30″ N, 08 35′04″ W). The site was a 10 ha sedimentation pond located in the industrial complex of Estarreja into which had been deposited over a 26 year period 300 000 tons of solid waste residues from the production of acetylene and polyvinyl chloride. The sediment had an electrical conductivity of 5980 μS cm^{-1}, 4.12% total organic C, 0.23% total N, 1.27% total Ca, 664.2 mg kg^{-1} total Na and 11 mg kg^{-1} Olsen's P (Oliveira et al. 2005a). The pH (H$_2$O) values of the sediment were found to be very high (11.8 and 12.6 at depths of 5 cm and 15 cm, respectively).

The two studied native plant species were *Salix atrocinerea* Brot. and *Conyza bilbaoana* J. Rémy, the dominant shrub and herbaceous plant species, respectively, at the study site (Oliveira et al. 2001, 2005b). Seeds of *C. bilbaoana*

and cuttings of *S. atrocinerea* were collected on the study site in order to obtain plants of local provenance and which were adapted to the alkaline sediment.

The four AMF used in this study were *Glomus intraradices* BEG163, *Glomus mosseae* BEG198, *Glomus geosporum* BEG199 and *Glomus claroideum* BEG210. These species of AMF were isolated as pure cultures derived from trap cultures established with field-collected roots and alkaline sediment (Oliveira et al. 2005b) and represent the majority of the species found at the site (Oliveira et al. 2005b). For a period of 12 months prior to the beginning of the experiment each species of AMF was grown separately in multispore pot culture with both *Zea mays* L. and *Trifolium pratense* L. as host plants in a 1:2 (v/v) mixture of zeolite (clinoptilolite 1.0–2.5 mm, Chemko, SK) and sterilised alkaline sediment from the site (autoclaved twice at 121°C for 25 min on two consecutive days). All AMF were propagated at the same time under the same greenhouse conditions.

Experimental design and set-up

Seeds of *C. bilbaoana* were surface sterilised with 0.5% (v/v) NaOCl for 5 min and pre-germinated in plastic trays containing a mixture of fine sand and attapulgite clay (Agsorb 8/16, Oil-Dry Ltd., Wisbech, UK) (1:1) pre-autoclaved twice (121°C for 25 min) on two consecutive days. Trays were placed in a controlled greenhouse with 16 h photoperiod, with supplementary metal halide 400 W lighting, 600–800 μmol m^{-2} s^{-1} photosynthetically active radiation. Temperature and relative humidity varied between 15 and 39 °C and 60 and 85%, respectively. All cuttings of *S. atrocinerea* were collected from shoots of the same plant in order to maintain genetic homogeneity. They were trimmed to 5 cm length and cuttings with 9 mm diameter were selected and washed four times with tap water (in series of 15 cuttings in 500 ml), surface sterilised twice with 0.5% (v/v) NaOCl for 25 min and rinsed three times with sterile deionised water for 10 min. Each cutting was placed individually in a 25 ml glass tube containing 10 ml of sterile deionised water. After 35 days, seedlings of *C. bilbaoana* of a similar size

and equally developed cuttings of *S. atrocinerea* were transplanted into Perspex rhizoboxes.

The rhizoboxes ($11 \times 10 \times 10.5$ cm) were divided into two root compartments (one at each end) ($4.5 \times 10 \times 10.5$ cm) and one hyphal compartment (HC) ($2 \times 10 \times 10.5$ cm) in the middle. The root compartments were separated from the hyphal compartment by inserts with a hole (7×6 cm) sealed with a nylon mesh (Silkprogress Ltd., Moravská Chrastová, CZ) with openings of 35 µm in diameter. The size of the openings allowed the hyphae of AMF but not the roots to grow from the root compartments to the central hyphal compartment to help obtain root-free samples of the ERM. All compartments of the rhizoboxes were filled with sterilised alkaline sediment (autoclaved twice at 121°C for 25 min on two consecutive days) collected on the site from the uppermost 5 cm layer and sieved through a 3 mm mesh. Microbial populations from the original non-sterile sediment were reintroduced to the autoclaved sediment by adding 50 ml of filtrate (Whatman No. 1) to the entire soil volume in all containers of each rhizobox. The filtrate was obtained from 300 g of sediment shaken for 2 h in 3 l of sterile deionised water.

One side of the rhizobox was planted with one seedling of *C. bilbaoana*, while the opposite side was planted with one rooted cutting of *S. atrocinerea*. The experiment comprised 6 treatments: (i) non-inoculated controls, (ii) plants inoculated with *Glomus intraradices* BEG163, (iii) *Glomus mosseae* BEG198, (iv) *Glomus claroideum* BEG210, (v) *Glomus geosporum* BEG199 and (vi) plants inoculated with a mixture of the four AMF. All treatments were replicated five times. Within each treatment, both plant species were inoculated simultaneously with the same AMF species or mixture of species. At transplanting, each rhizobox received 20 g (10 g in each root compartment) of the corresponding inoculum consisting of colonised root fragments, hyphae and spores in the growth substrate described above, placed 2 cm below the root systems. Plants inoculated with the mixture of AMF received an inoculum composed of equal parts of the four inocula. Non-inoculated controls received the same inocula mixture autoclaved twice (121°C for 25 min) on two consecutive days. Twenty ml of a filtrate of the AMF inocula mixture were added to the entire soil volume in all containers of each rhizobox to eliminate differences in microbial populations introduced with the AMF inocula (Koide and Li 1989). This inocula filtrate was prepared as described above for the sediment. The plants were grown in the same controlled greenhouse described above and watered every 2 days with deionised water. The rhizoboxes with different inoculation treatments were periodically rotated to different bench positions to minimise differences due to their location in the greenhouse.

Plant parameters analyses

After a growth period of 8 months the root systems were separated from the shoots and washed to remove adhered sediment. A fresh sub-sample (0.5 g) of roots of *C. bilbaoana* and *S. atrocinerea* was collected to assess arbuscular mycorrhizal colonisation (described below). The remaining root system was weighed and dried at 70°C for 48 h together with the shoot. The dried root system and shoot were then re-weighed. The dry root mass of the sub-sample was calculated by multiplying its fresh mass by the dry to fresh mass ratio of the root system. The sum of the dry mass of the root sub-sample with the dry mass of the root system and the dry mass of the shoot gave the total dry weight per plant. The number of seed spikes per plant of *C. bilbaoana* was counted before drying the plant material. The coexistence ratio was determined by dividing the biomass of *C. bilbaoana* by the sum of the biomasses of *C. bilbaoana* and *S. atrocinerea*. The mycorrhizal dependency of *C. bilbaoana* and *S. atrocinerea* was calculated according to the equations presented by van der Heijden (2002) encompassing all AMF treatments. Oven-dried leaves of *C. bilbaoana* and *S. atrocinerea* were finely ground and 0.3 g of material was digested according to Novozamsky et al. (1983). The phosphorus (P) concentration in leaves was determined by colorimetry (Helios Gamma,Unicam, Cambridge, UK) (Walinga et al. 1989).

Fungal parameters analyses

At harvest, a sub-sample of fresh roots of *C. bilbaoana* and *S. atrocinerea* was cut into 1-cm pieces and stained using a modified Phillips and Hayman (1970) protocol (Oliveira et al. 2005b). Percentage root length colonised (% RLC) by AMF was assessed for each plant species by using a gridline intersect method (Giovannetti and Mosse 1980) under a stereomicroscope (SZ60, Olympus, Tokyo, Japan). Stained root pieces were mounted on glass slides and examined with a compound microscope (BX60, Olympus, Tokyo, Japan) (×100–400) to assess the abundance of arbuscules in the mycorrhizal root segments (Trouvelot et al. 1986). The arbuscule abundance was expressed as the percentage of the colonised root length occupied by arbuscules. The sediment from the HC was homogenised after plant growth and the ERM length was evaluated by a modified membrane filtration technique (Jakobsen et al. 1992). The total length of ERM was assessed using the grid-line intersect method under a compound microscope, with an ocular grid at ×200 magnification (Brundrett et al. 1994). The background length of mycelium found in the non-mycorrhizal treatment was subtracted from the values obtained in the mycorrhizal treatments. The result was expressed in cm of hyphae per 1 g of air-dried sediment. The viability of the ERM was evaluated by NADH diaphorase activity (Sylvia 1988) in the remaining ERM extracted from the sediment in the HC by wet-sieving. The proportion of ERM length that contained purple precipitate (NADH diaphorase activity) was estimated under a compound microscope at ×200 magnification.

Statistical analysis

All the data were analysed using one-way analysis of variance (ANOVA). When a significant *F*-value was obtained ($P < 0.05$), treatment means were compared using the Duncan's multiple range test. The data from the fungal parameters were analysed without including the non-inoculated control treatment. Regression analysis between coexistence ratio and ERM length was conducted without including the non-inoculated control treatment. In order to distinguish between the effect of AMF treatment and ERM length on the coexistence ratio, an analysis of covariance (ANCOVA) was performed using ERM length as the covariate. ANCOVA was conducted at a significance level of 0.05 without including the non-inoculated control treatment. All statistical analyses were performed using SPSS 11.0.0 software package (SPSS Inc., Chicago, IL, USA).

Results

All plants of *C. bilbaoana* and *S. atrocinerea* in the mycorrhizal treatments were colonised by the native AMF (Table 1). No AMF colonisation was observed in non-inoculated control rhizoboxes. The % RLC of *C. bilbaoana* was significantly greater in rhizoboxes inoculated with *G. intraradices* BEG163, *G. geosporum* BEG199 or the mixed inocula compared with the other treatments. There were no significant differences in % RLC between *S. atrocinerea* plants in the mycorrhizal treatments. *Conyza bilbaoana* always had high levels of % RLC (between 71 and 89%), whereas *S. atrocinerea* supported low levels (4 or 5%).

There was a significant variation in arbuscule abundance in the mycorrhizal root segments of *C. bilbaoana* and *S. atrocinerea* among the different AMF treatments (Table 1). The arbuscule abundance in the mycorrhizal root segments of *C. bilbaoana* was significantly greater in plants inoculated with the mixed inocula, followed by plants inoculated with *G. intraradices* BEG163 and *G. claroideum* BEG210. The arbuscule abundance in the mycorrhizal root segments of *S. atrocinerea* was significantly greater in plants inoculated with *G. claroideum* BEG210, followed by plants inoculated with *G. intraradices* BEG163 and the mixed inocula.

There were significant differences in the ERM length and NADH diaphorase activity in the hyphal compartment of the rhizoboxes among the different AMF treatments (Table 2). The ERM length associated with the roots of both plant species was significantly greater in rhizoboxes inoculated with *G. mosseae* BEG198, *G. claroideum* BEG210 or the mixed inocula. The NADH

Table 1 Percentage root length colonised (% RLC) by AMF and arbuscule abundance (percentage of the colonised root length occupied by arbuscules) of *Conyza bilbaoana* and *Salix atrocinerea* at harvest

Treatment	*Conyza bilbaoana*		*Salix atrocinerea*	
	AMF colonisation (%RLC)	Arbuscules (%)	AMF colonisation (%RLC)	Arbuscules (%)
Glomus intraradices BEG163	82 ± 3 b	71 ± 2 c	5 ± 0.8	53 ± 3 c
Glomus mosseae BEG198	75 ± 2 a	11 ± 1 a	4 ± 0.4	13 ± 2 a
Glomus claroideum BEG210	71 ± 2 a	45 ± 2 b	5 ± 0.5	85 ± 2 d
Glomus geosporum BEG199	87 ± 3 b	14 ± 2 a	4 ± 0.5	15 ± 2 a
Mix	89 ± 3 b	89 ± 2 d	5 ± 0.6	44 ± 2 b
F-value of one-way ANOVA	$F_{4,20} = 9^{***}$	$F_{4,20} = 355^{***}$	$F_{4,20} = 0.8$ NS	$F_{4,20} = 161^{***}$

Means of five observations (±1 SE) followed by the same letters within each column are not significantly different according to Duncan's multiple range test at the level of $P < 0.05$. *** $P < 0.001$, NS non-significant effect

diaphorase activity of the ERM was significantly greater in rhizoboxes inoculated with *G. mosseae* BEG198 or the mixed inocula.

There were no significant differences in total biomass per rhizobox (sum of the biomasses of both plant species) between non-inoculated controls and plants inoculated with AMF (Fig. 1). However, different AMF treatments had significantly different effects on the biomass partitioning between both plant species (Fig. 2a). *Glomus intraradices* BEG163, *G. mosseae* BEG198, *G. claroideum* BEG210 and the mixed inocula significantly increased the total biomass of *C. bilbaoana* while decreasing the total biomass of *S. atrocinerea* compared with non-inoculated control plants. *Glomus geosporum* BEG199 significantly increased total biomass of *S. atrocinerea*, but failed to promote the growth of *C. bilbaoana*.

Table 2 Extraradical mycelium length and NADH diaphorase activity in the hyphal compartment of the rhizoboxes at harvest

Treatment	ERM length (cm g^{-1})	NADH diaphorase activity (%)
Glomus intraradices BEG163	315 ± 34 a	38 ± 4 a
Glomus mosseae BEG198	542 ± 148 b	61 ± 4 cd
Glomus claroideum BEG210	558 ± 75 b	44 ± 3 ab
Glomus geosporum BEG199	271 ± 46 a	52 ± 7 bc
Mix	633 ± 37 b	68 ± 1 d
F-value of one-way ANOVA	$F_{4,18} = 5^{**}$	$F_{4,20} = 8^{**}$

Means of five observations (± 1 SE) followed by the same letters within each column are not significantly different according to Duncan's multiple range test at the level of $P < 0.05$. ** $P < 0.01$

The mycorrhizal dependency of *C. bilbaoana* and *S. atrocinerea*, calculated encompassing all AMF treatments was 56 and –44%, respectively.

The leaf P concentrations in both plant species differed significantly among the different AMF treatments (Fig. 2b). *Glomus claroideum* BEG210 and the mixed inocula significantly increased the leaf P concentrations of *C. bilbaoana*, but did not significantly influence that of *S. atrocinerea*. *Glomus intraradices* BEG163, *G. mosseae* BEG198 and *G. geosporum* BEG199 significantly increased the leaf P concentration of both *C. bilbaoana* and *S. atrocinerea* compared with the respective non-inoculated control plants.

The AMF treatments had significantly different effects on the coexistence ratio between *C. bilbaoana* and *S. atrocinerea* (Fig. 3). *Conyza*
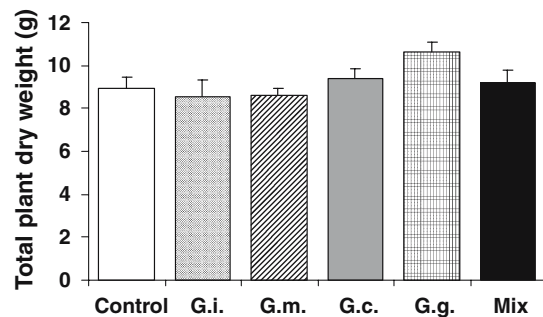

Fig. 1 Total dry weight of both plant species (*Conyza bilbaoana* and *Salix atrocinerea*) in each rhizobox, non-inoculated, inoculated with *Glomus intraradices* BEG163 (G.i.), *Glomus mosseae* BEG198 (G.m.), *Glomus claroideum* BEG210 (G.c.), *Glomus geosporum* BEG199 (G.g.) and plants inoculated with a mixture of the four AMF (Mix). Values are means of five observations ± 1 SE. The *F*-value of one-way ANOVA was $F_{5,24} = 2.1$ ($P > 0.05$)
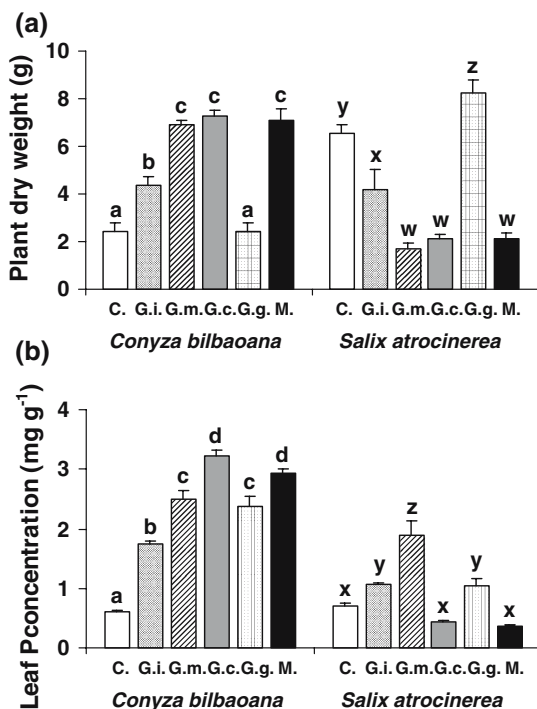
(a)

(b)

Fig. 2 Total dry weight (**a**) and leaf phosphorus concentration (**b**) of *Conyza bilbaoana* and *Salix atrocinerea* non-inoculated (C.), inoculated with *Glomus intraradices* BEG163 (G.i.), *Glomus mosseae* BEG198 (G.m.), *Glomus claroideum* BEG210 (G.c.), *Glomus geosporum* BEG199 (G.g.) and plants inoculated with a mixture of the four AMF (Mix). Values are means of five observations ±1 SE. The *F*-values of one-way ANOVA for *C. bilbaoana* and *S. atrocinerea* dry weight were $F_{5,24} = 46.8$ ($P < 0.0001$) and $F_{5,24} = 33.0$ ($P < 0.0001$), respectively. The *F*-values of one-way ANOVA for *C. bilbaoana* and *S. atrocinerea* leaf phosphorus concentration were $F_{5,24} = 75.0$ ($P < 0.0001$) and $F_{5,24} = 23.5$ ($P < 0.0001$), respectively. Columns marked with different letters within each plant species differed significantly according to Duncan's Multiple Range test at $P < 0.05$

bilbaoana had a greater proportional biomass in rhizoboxes inoculated with *G. intraradices* BEG163, *G. mosseae* BEG198, *G. claroideum* BEG210 or the mixed inocula, than in non-inoculated controls. However, in rhizoboxes inoculated with *G. geosporum* BEG199 and in non-inoculated controls *S. atrocinerea* had a significantly greater proportional biomass than *C. bilbaoana*.

Only in the rhizoboxes inoculated with *G. mosseae* BEG198, *G. claroideum* BEG210 or the mixed inocula, did *C. bilbaoana* plants produce seed spikes (Fig. 4). The rhizoboxes with the
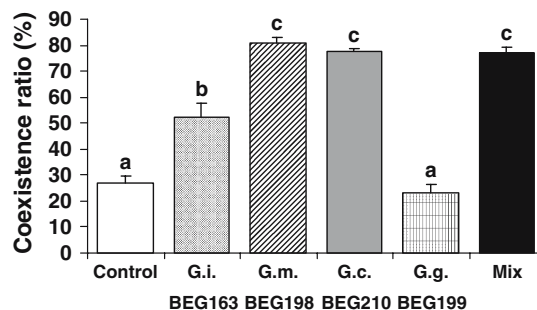

Fig. 3 Coexistence ratio between *Conyza bilbaoana* and *Salix atrocinerea* in rhizoboxes non-inoculated, inoculated with *Glomus intraradices* BEG163 (G.i.), *Glomus mosseae* BEG198 (G.m.), *Glomus claroideum* BEG210 (G.c.), *Glomus geosporum* BEG199 (G.g.) and plants inoculated with a mixture of the four AMF (Mix). The coexistence ratio expresses the biomass of *C. bilbaoana* as a percentage of the total biomass of *C. bilbaoana* and *S. atrocinerea*. Values are means of five observations ±1 SE. The *F*-value of one-way ANOVA was $F_{5,24} = 74.9$ ($P < 0.0001$). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$
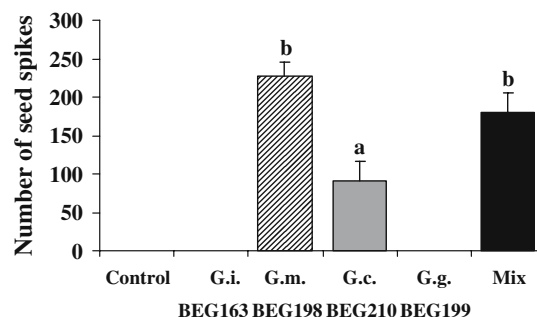

Fig. 4 Number of seed spikes per plant produced by *Conyza bilbaoana* in rhizoboxes non-inoculated, inoculated with *Glomus intraradices* BEG163 (G.i.), *Glomus mosseae* BEG198 (G.m.), *Glomus claroideum* BEG210 (G.c.), *Glomus geosporum* BEG199 (G.g.) and plants inoculated with a mixture of the four AMF (Mix). Values are means of five observations ±1 SE. The *F*-value of one-way ANOVA was $F_{2,12} = 9$ ($P < 0.01$). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$

most seed spikes produced by *C. bilbaoana* were the ones inoculated with *G. mosseae* BEG198 or the mixed inocula.

There was a strong significantly positive correlation between the coexistence ratio of both plant species and the ERM length of AMF extracted from the HC (Fig. 5). Species of AMF with high amounts of ERM in the HC were found
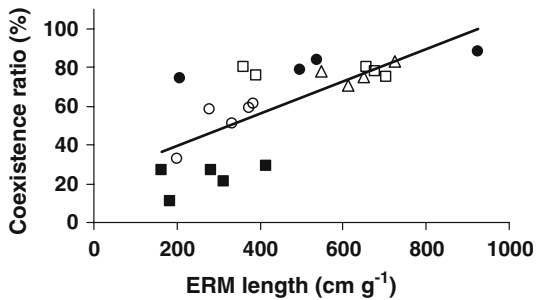
Fig. 5 Relationship between plant coexistence ratio and the extraradical mycelium length in the hyphal compartment of the rhizoboxes, $y = 0.083x + 23$, $R^2 = 0.514$, $P < 0.001$. *Glomus intraradices* BEG163 (open circles), *Glomus mosseae* BEG198 (closed circles), *Glomus claroideum* BEG210 (open squares), *Glomus geosporum* BEG199 (closed squares), mixture of the four AMF (open triangles)

to promote the dominance of *C. bilbaoana*. Conversely, the AMF species with less developed ERM in the HC were found to promote the dominance of *S. atrocinerea*.

The *F*-values of ANCOVA for ERM length and AMF treatment were $F_{1,17} = 4.9$ ($P < 0.05$) and $F_{4,17} = 30.9$ ($P < 0.0001$), respectively. This shows that after considering the effect of AMF treatment on the coexistence ratio, there is still a significant correlation between the coexistence ratio of both plant species and the ERM length of AMF. The results from ANCOVA also show that the effect of the AMF treatment on the coexistence ratio is significant after accounting for the variation explained by the ERM length.

Discussion

Different AMF species or the absence of AMF did not influence total plant productivity (sum of the biomasses of both plant species), but had a great impact on the individual biomass of *C. bilbaoana* and *S. atrocinerea*. The coexistence between *C. bilbaoana* and *S. atrocinerea* was changed according to the AMF species present. Similar results were obtained by van der Heijden et al. (2003) in a study with a grass and a forb, where different AMF species influenced how the plants coexisted.

In the present work, the high % RLC in *C. bilbaoana* (between 71 and 89%) and the low

levels in *S. atrocinerea* (4 or 5%) were similar to those observed for these plant species growing at the study site (Oliveira et al. 2001; 2005b). Van der Heijden (2001) reported low levels of % RLC (5%), but enhanced growth of the plant compared with non-colonised controls in a study with *Salix repens* cuttings obtained from sand dunes and inoculated with *G. mosseae* in a laboratory microcosm experiment. This shows that even with low levels of % RLC, species of *Salix* can greatly benefit from the AMF symbiosis.

Conyza bilbaoana had a high mycorrhizal dependency (56%) and *S. atrocinerea* had a low mycorrhizal dependency (–44%), calculated using the mean plant dry masses of all AMF treatments. A mycorrhizal dependency > 0 means that a plant benefits from AMF, while a mycorrhizal dependency < 0 means that AMF reduces the growth of a plant under the prevailing environmental conditions (van der Heijden 2002). Thus, generally, the growth of *C. bilbaoana* benefited from the mycorrhizal symbiosis, while AMF inhibit the growth of *S. atrocinerea* at this stage of its development. However, if only *G. geosporum* BEG199 was considered, the mycorrhizal dependency of *C. bilbaoana* and *S. atrocinerea* would have been 0% and 21%, respectively, indicating that this fungus had a neutral growth effect on the former plant species and a beneficial growth effect on the latter plant species. This shows that the identity of the AMF can determine how plant species coexist and the magnitude of plant growth response can be very high according to the AMF species present, ranging from negative to positive. In our study we used a dominant (frequently found) plant species with a high mycorrhizal dependency (*C. bilbaoana*) and a subordinate (less frequently found) plant species with a low mycorrhizal dependency (*S. atrocinerea*) and according to the prediction of the conceptual model proposed by Urcelay and Díaz (2003), the outcome of the presence of AMF should be a decrease in plant species diversity, by enhancing the competitive ability of the dominant and negatively influencing the coexistence of both plant species. For simplification purposes, their model includes only two levels of AMF (absence or presence), however, our results indicate that there can be different effects in plant species

coexistence (both the dominant or the subordinate plant species can be preferentially favoured) depending on the AMF species present. Therefore, not only absence or presence of AMF, but also the composition of AMF communities must be taken into account in determining the effect of AMF on plant coexistence and diversity (van der Heijden et al. 1998b; 2003). This may be particularly relevant in early succession ecosystems (e.g. anthropogenic sites), with patchy distributions of AMF populations and their host plants, like those studied in the highly alkaline industrial sediment (Oliveira et al. 2005b).

The AMF treatments where higher coexistence ratios were observed (*G. mosseae* BEG198, *G. claroideum* BEG210 and the four mixed AMF) were also the ones where greatest ERM lengths were found in the HC. The results show that AMF species with greater ERM lengths away from the rhizosphere preferentially benefited the plant species with a high mycorrhizal dependency (*C. bilbaoana*), while the AMF species with the lowest ERM length in the HC benefited the plant species with a low mycorrhizal dependency (*S. atrocinerea*). The observed growth benefit in *C. bilbaoana* could be attributed to increased phosphate uptake, except in plants inoculated with *G. geosporum* BEG199. AMF species with greater ERM lengths can have better phosphate uptake efficiencies due to the large surface area of the mycelium used for scavenging the soil (Jakobsen et al. 1992; Leake et al. 2004; Schweiger and Jakobsen, 2000). It is, therefore, possible that the greater ERM lengths of *G. mosseae* BEG198, *G. claroideum* BEG210 and that resulting from the four mixed AMF may have contributed to the greater proportional biomass of *C. bilbaoana* through an increased phosphate uptake. We obtained no direct relationship between ERM length and viability (evaluated by NADH diaphorase activity). However, the NADH diaphorase activity of the ERM of *G. mosseae* BEG198, *G. claroideum* and the four mixed AMF was high, indicating that a large proportion of the hyphae was alive. The reasons for the AMF species with smaller ERM length preferentially benefiting the growth of *S. atrocinerea* are less clear and could not be attributed to the observed plant P levels. An expansive

ERM may represent a higher drain of photosynthate (Hart and Reader 2002) and it is possible that *S. atrocinerea* was less tolerant to carbon drain than *C. bilbaoana*, especially under reduced light levels (artificial light compared with natural light) (Graham and Eissenstat 1994; 1998). This could, thus, explain the observed growth depression of *S. atrocinerea* inoculated with the AMF species with the greater ERMs (*G. mosseae* BEG198, *G. claroideum* BEG210 and the four mixed AMF).

It is possible that *C. bilbaoana* and *S. atrocinerea* were linked by means of ERM (Simard et al. 2002) spreading from the roots of one plant species, across the hyphal compartment and colonising the roots of the plant species in the opposite compartment; and by means of anastomoses (Giovannetti et al. 2004) between the hyphae of the ERM originating from each of the plant species. These interplant mycorrhizal hyphal connections may have allowed the transfer of plant-derived carbon between the two plants (Fitter et al. 1998; Graves et al. 1997). Van der Heijden (2002) showed that interplant carbon transfer through mycorrhizal hyphal connections is directed towards plant species with the higher mycorrhizal dependencies. Therefore, carbon transfer from *S. atrocinerea* to *C. bilbaoana* and consequent growth depression of *S. atrocinerea*, could have occurred to a greater extent in the treatments where a larger ERM development was observed. However, the relevance of these interplant carbon transfer mechanisms for plant coexistence is still controversial (Simard et al. 2002), since recent studies have indicated that the transferred carbon was not transported to the shoots of the receiver plant (Fitter et al. 1998) and it likely remained in the intraradical mycelium (Pfeffer et al. 2004) being, therefore, unavailable to the receiver plant.

In the present work, *G. geosporum* BEG199 produced the smallest ERM away from the rhizosphere when compared with the other species of *Glomus* studied, even though similar percentages of root colonisation were obtained in the different AMF treatments. Similar observations were reported by Green et al. (1994) in a study with another *G. geosporum* isolate and two other species of *Glomus*. The acquisition of phosphate

requires an extensive ERM developed away from the rhizosphere (beyond the phosphate depletion zone), whereas functions like soil aggregation to roots requires an ERM developed closer to roots (Fitter 2005). Therefore, *G. geosporum* BEG199 could have improved the growth of *S. atrocinerea* through an effect related to its pattern of ERM development (Dodd et al. 2000). Apart from phosphate acquisition, AMF have several other beneficial effects on plants, such as improved water relations by binding roots to soil (Wright and Upadhyaya 1998), increased resistance to soil-borne plant pathogens (Newsham et al. 1995), uptake of other nutrients (Smith and Read 1997), alleviation of stresses caused by high soil salinity and pH (Oliveira et al. 2005a; Sylvia and Williams 1992), among others, and since some of these effects can be AMF species specific, it is possible that *G. geosporum* BEG199 has improved preferentially the growth of *S. atrocinerea* in the highly alkaline sediment, indirectly through some of these effects.

Our experiment was conducted with only one genotype of *S. atrocinerea* and similar studies on the effects of AMF on growth of this plant species and its coexistence with *C. bilbaoana* using different genotypes would help to broaden the conclusions taken in this study.

Variation in ERM length of AMF accounted for a large proportion (51.4%) of the variation in the coexistence ratio between *C. bilbaoana* and *S. atrocinerea*, indicating that the length of the ERM of AMF can influence how these two plant species can coexist in a highly alkaline anthropogenic sediment under the experimental conditions employed in this study.

Despite the importance of AMF to plant coexistence (Hart et al. 2003, van der Heijden et al. 2003) little is known about the effect of AMF colonisation on the reproduction of coexisting plant species. In our study, *C. bilbaoana* without AMF or when colonised by *G. intraradices* BEG163 or *G. geosporum* BEG199 did not produce seed and therefore could not reproduce. The AMF treatments where *C. bilbaoana* produced seed spikes were among the treatments where a greater plant biomass and P concentration was observed, indicating that seed production could have been related to an improved phosphate uptake by some AMF species (Helgason et al. 2002; Jakobsen et al. 1992; Jansa et al. 2005; Munkvold et al. 2004). One of the reasons why different species of AMF can differ in the way they provide phosphate to plants might be related to their lifecycle requirements from the symbiosis. AMF may be able to retain phosphate in the mycelium and regulate its transfer to the plant so as to receive sufficient carbon for the formation of spores over a longer period of time (Dodd et al. 2000). In experiments using tropical forage legumes, Boddington and Dodd (1998) compared the development of the mycelium of three species of AMF and the localisation and activity of the vacuolar alkaline phosphatase in their mycelia. They suggested that different AMF species have different mechanisms for the control of P transfer, within the mycelium, to the host. Our results suggest that AMF may be playing a very important role in the reproduction of *C. bilbaoana* coexisting with *S. atrocinerea* in the alkaline sediment and that not only the absence or presence of AMF, but also the species identity has the potential to stimulate or completely inhibit seed production and, therefore, reproduction. Given that the native AMF can prevent or stimulate *C. bilbaoana* to reproduce and given that *C. bilbaoana* is the dominant plant species at the study site, the identity of the AMF present could have a strong influence on the plant community structure (van der Heijden et al. 1998a). However, long-term studies would be necessary to demonstrate this hypothesis unequivocally.

An increase in AMF diversity from one species to four did not increase total plant productivity and therefore it is unlikely that functional complementarity in phosphate uptake among the different AMF species occurred (Hart and Klironomos 2002; Koide 2000a). No significant differences in biomass were observed between *C. bilbaoana* inoculated with the four AMF and inoculated with the most beneficial single AMF species. Similar results were obtained by van der Heijden et al. (1998b) in an experiment simulating European calcareous grassland using eleven plant species. *Glomus geosporum* BEG199, the most beneficial AMF species for *S. atrocinerea*, was present in the mixed inocula and yet no growth improvement of *S. atrocinerea* was

observed in the mixed inocula treatment. Competition for *S. atrocinerea* root niche between the AMF species in the mixed inocula could have occurred (Pearson et al. 1993). *Glomus geosporum* BEG199 was probably outcompeted by the other AMF and this may have contributed to the lack of increase in plant productivity.

This study shows that native AMF species identity can influence the coexistence between two co-occurring plant species in a highly alkaline anthropogenic sediment and that this influence is related with the length of the ERM of AMF. The identity of the native AMF species also showed a strong impact on the reproduction of the dominant plant species. These findings are relevant not only to plant and mycorrhizal ecology in extreme anthropogenic ecosystems, but also for the application of a mycorrhizal fungi based phytorestoration strategy on the highly alkaline sediment, since promoting plant species coexistence will contribute to the re-establishment of sustainable plant ecosystems.

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