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Mycorrhizal community resilience in response to experimental plant functional type removals in a woody ecosystem

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Summary

1. Dominant plant functional types (PFTs) are expected to be primary determinants of communities of other above- and below-ground organisms. Here, we report the effects of the experimental removal of different PFTs on arbuscular mycorrhizal fungi (AMF) communities in a shrubland ecosystem in central Argentina.

2. On the basis of the biomass-ratio hypothesis and plant resource use strategy theory, we expected the effect of removal of PFTs on AMF colonization and spores to be proportional to the biomass removed and to be stronger when more conservative PFTs were removed. The treatments applied were: undisturbed control (no plant removed), disturbed control (mechanical disturbance), *no shrub* (removal of deciduous shrubs), *no perennial forb* (removal of perennial forbs), *no graminoid* (removal of graminoids) and *no annual forb* (removal of annual forbs). AMF colonization was assessed after 5, 17 and 29 months. Total density of AMF spores, richness and evenness of morphotaxa, and AMF functional groups were quantified after 5, 17, 29, 36 and 39 months.

3. Five months after the initial removal we found a significant reduction in total AMF colonization in all plots subjected to PFT removals and in the disturbed control plots, as compared with the undisturbed controls. This effect disappeared afterwards and no subsequent effect on total colonization and colonization by arbuscules was observed. In contrast, a significant increase in colonization by vesicles was observed in months 17 and 29, mainly in *no graminoid* plots. In general, treatments did not significantly affect AMF spores in the soil. On the other hand, *no annual forb* promoted transient (12–18 months) higher ammonia availability, and *no shrub* promoted lower nitrate availability in the longer term (24–28 months).

4. *Synthesis*. Our experiment, the first to investigate the effects of the removal of different PFTs on AMF communities in natural ecosystems, indicates that AMF communities are resilient to changes in the soil and in the functional composition of vegetation. Furthermore, it does not provide consistent evidence in support of the biomass-ratio hypothesis or differential trait-based direct or indirect effects of different PFTs on AMF in this particular system.

Key-words: arbuscular mycorrhizal fungi, Argentina, biomass-ratio hypothesis, community-reduction experiment, functional diversity, plant functional types, plant traits, removal experiment

Introduction

The extent to which plant species or groups of species affect the functioning of local ecosystems is likely to depend on their traits and their contribution to total biomass (Grime 1998, 2006; Diaz *et al*. 2004; Garnier *et al*. 2004). Accordingly, dominant plant functional types (PFTs), that is, groups of plants that share similar traits and therefore show similar responses to environmental constraints and/or similar effects on main ecosystem processes (Díaz & Cabido 1997; Lavorel *et al*. 1997)

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should be the main drivers of carbon (C), nutrient and water cycling in ecosystems. They should also be major determinants of communities of other above- and below-ground organisms (Wardle 2002). Within below-ground communities, arbuscular mycorrhizal fungi (AMF) represent a fundamental component in most terrestrial ecosystems. AMF often benefit plants by improving phosphorus (P) acquisition, protecting roots against pathogens, alleviating water stress and enhancing competitive ability (Wardle *et al.* 2004). AMF form a variety of structures with different function, morphology and longevity. Intraradical hyphae and arbuscules are sites for nutrient transfer between the symbionts (Smith & Read 2008). Lipids produced on the basis of C from plant photosynthesis are transported through the intra- and extraradical mycelium and stored in high concentrations in intraradical vesicles and extraradical spores (Bago, Pfeffer & Shachar-Hill 2002; Smith & Read 2008). Depending on the balance between costs and benefits to the plant, AMF range from mutualists to parasites (Johnson, Graham & Smith 1997) and have different effects on species richness and dominance hierarchies of plant communities (see Hartnett & Wilson 2002; van der Heijden 2002; Urcelay & Díaz 2003 for reviews). Conversely, much less is known about the effect of vegetation on AMF. Host plant species can affect AMF communities directly by regulating the allocation of C to roots and indirectly by changing soil environmental conditions (Johnson *et al.* 2005). Differential AMF response to, or preference for, different host plants has been shown using spore (Johnson, Tilman & Wedin 1992; Bever *et al.* 1996; Eom, Hartnett & Wilson 2000; Castelli & Casper 2003; Lovelock, Andersen & Morton 2003; Lovelock & Ewel 2005) and molecular (Helgason *et al.* 2002; Vandenkoornhuyse *et al.* 2002, 2003) information. Experiments with synthetically assembled communities have shown that plant species richness affects AMF spore number and volume (Burrows & Pfleger 2002a; Burrows and Pfleger 2002b; Chen *et al.*2004), specific phospholipid fatty acids (Hedlund *et al.* 2003) and diversity of AMF gene sequences in phytometer roots (Johnson *et al.* 2004). These changes can potentially feed back on plant communities affecting the coexistence and dominance of their component species (Bever 2003). However, community- and ecosystem-level patterns observed in highly controlled synthetic systems often differ from those in natural systems (Díaz *et al.* 2003). Despite this recognition, to our knowledge no study has specifically addressed the effects of experimental manipulation of plant community composition on AMF communities in natural ecosystems.

In the present study, we asked what the effects of removing dominant PFTs (deciduous shrubs, perennial forbs, graminoids and annual forbs) were on AMF colonization and spore density and diversity in a natural mountain shrubland of central Argentina. We hypothesized that in the longer term the removal of different PFTs should have differential effects on AMF colonization and spore community parameters and that those effects should (a) be proportional to the biomass removed; and (b) be significantly related to the general resource use strategy of the PFTs in question. Our hypotheses and predictions were based on two general, not mutually exclusive, bodies of theory. On the one hand, the existence of general plant resource use strategies, that is, plants from different floras, ecosystems and clades, show consistent trait syndromes leading to conservation of resources or to rapid acquisition, use and release (Aerts & Chapin 2000; Grime 2001; Diaz *et al.* 2004; Wright *et al.* 2004); this includes mycorrhizal dependence, which tends to be weaker in plant groups with acquisitive syndromes (e.g. high fine root production) such as annual forbs and fast-growing grasses (Brundrett 1991). Moreover, our prediction is based on the empirical evidence that shows that C3 grasses and annual forbs were less mycorrhiza-dependent and showed lowest mycorrhizal colonization rates than perennial dicots (Wilson & Hartnett 1988). A corollary of this hypothesis is that different PFTs exploit different resources, therefore no important compensation by other PFTs should be observed after removals (Walker 1992). In addition, according to the biomass-ratio hypothesis (Grime 1998), the effects of plants on ecosystem and community processes should be proportional to their local abundance. This is expected to apply in the case of mycorrhizal communities, especially considering that AMF are obligate biotrophs that depend on plant photosynthetic products for their survival (Smith & Read 2008). Therefore, in the relatively long time frame of this study, the decrease of the C supply as a consequence of biomass reduction should negatively impact the spore communities. This prediction is also supported by previous evidence of the expected positive relationship between plant biomass and AMF spore production and colonization (Burrows & Pfleger 2002b; Chen *et al.* 2004, 2005). According to these not mutually exclusive hypotheses, we predicted that removal of PFTs would negatively affect AMF colonization, spore density and diversity according to this decreasing order: removal of deciduous shrubs, perennial forbs, graminoids and annual forbs. Although we expected that the response patterns of the different AMF variables to removals would vary in the same direction, this does not imply a necessary direct causal link between them.

Although field removal experiments involve conditions that are not as well controlled as those in synthetic-assemblage experiments, they are more useful for understanding the behaviour of natural ecosystems, in particular the effects and responses of comparatively large- and slow-growing species, such as shrubs and trees, and the effects of complex interactions between organisms belonging to different trophic levels (Díaz *et al.* 2003). In the specific case of links between plants and below-ground organisms, the removal approach has recently shown to be successful in assessing the effects of the removal of perennial herbs on soil decomposers (Wardle *et al.* 1999), and those of the removal of shrubs on ectomycorrhizas (Urcelay *et al.* 2003; McHugh & Gehring 2006).

Materials and methods

SITE DESCRIPTION AND TREATMENTS

This study was conducted in a mountain shrubland in Sierras Grandes de Córdoba, central Argentina (31°30′ N, 64°35′ W, 880 m a.s.l.). Historic mean annual precipitation is 720 mm (de Fina 1992),

with most of the rainfall occurring in the warm season. The mean annual precipitation during the sampling period of this experiment (1998–2002) was 914 mm, ranging from 776 to 1095 mm. Historic mean annual temperature is 14.5 °C (de Fina 1992) and mean annual temperature during the experiment was 16.1 °C in 1999, 15.09 °C in 2000 and 17.95 °C in 2001. Soils are sandy, well drained and shallow (lithic Ustorthents Entisols). Chemical properties at the beginning of the experiment averaged: 7.01% $C_{organic}$, 2.85 mg g^{-1} N_{total}, 0.17 mg g^{-1} P_{total} and pH = 6.6. The experiment was established in a secondary shrubland dominated by the deciduous shrub *Acacia caven* (Mol.) Molina (Fabaceae), the perennial forb *Hyptis mutabilis* (Rich.) Briq (Lamiaceae), the C_3 grass *Stipa eriostachya* Kunth and the annual forb *Bidens pilosa* L (Asteraceae) (see Table S1 in Supporting Information). This is the most extensive vegetation type in the lower mountain slopes of central Argentina (Cabido & Zak 1999).

In November–December 1998, we established six blocks within a fenced area of *c*. 1 ha. We defined the blocks according to topography and the spatial heterogeneity of the vegetation. Each block consisted of six 4 × 4 m plots (36 plots in total). We applied treatments to whole plots but sampled only their 2 × 2 m inner portion to avoid edge effects. All plots were separated from each other by a distance of at least 1 m. Within each block the plots were randomly assigned to the following treatments: (i) undisturbed control (no plant removed), (ii) disturbed control (mechanical disturbance), (iii) *no shrub* (removal of deciduous shrubs), (iv) *no perennial forb* (removal of perennial forbs), (v) *no graminoid* (removal of graminoids, including grasses and sedges) and (vi) *no annual forb* (removal of annual forbs). The initial mean cover per plot of each PFTs was: deciduous shrubs: 81%; perennial forbs: 47%; graminoids: 33%; annual forbs: 17%. The average biomass (fresh weight per plot) removed at the beginning of the experiment was 3.6816 kg for deciduous shrubs, 6.608 kg for perennial forbs, 2.224 kg for graminoids and < 0.800 kg for annual forbs. Plants were removed by pulling out the above-ground stems and as much as possible of the below-ground structures. Care was taken to avoid breaking the stems and roots of non-target species. Shrubs and the few occurring large graminoids were cut off at the base of the stem because it was impossible to remove the complete root systems without causing major disturbance (see McLellan, Fitter & Law 1995). In the case of large tussock grasses, the crown with most of the ground-level meristems was also removed. In the case of shrubs, after cutting at the base we applied a systemic herbicide (Togar BT: Picloram 3% – Triclopir 6%; DowAgroSciences, Buenos Aries, Argentina) to the stumps with a brush, carefully avoiding neighbouring plants. After setting up the experiment in November 1998, we continued the removal operations throughout the whole experiment to maintain the plots free of target species that resprouted or germinated, although with time they were required with increasingly lower frequency. In the case of *A. caven*, the application of the herbicide on some stumps was repeated on a few occasions during approximately 1 year, during which the resprouting became increasingly weaker. After that, no further resprouting was observed. Therefore, we are certain that the vast majority of the root systems of the removed individuals was dead at the time of measurement, and thus could not have acted as a persistent source of C for AM communities. The disturbed control treatment was applied to simulate the side effects of removal and consisted of pulling the stems of the vegetation without breaking them and disrupting the surface soil layer by grabbing and shaking it. No biomass was removed from these plots.

SAMPLING

Five (April 1999), 17 (April 2000) and 29 (April 2001) months after the initial removals we took six soil cores of 5.5 cm diameter and up to 20 cm length, depending on the soil profile depth at each plot. These six subsamples were subsequently combined into a single compound sample per plot. From those samples, we extracted the roots and the AMF spores. In addition, we repeated soil sampling in November 2001 and February 2002 to investigate seasonal variations in spore communities. Thus, we obtained data on spore communities in autumn (April 2001), spring (November 2001) and summer (February 2002) that is 29, 36 and 39 months after initial removals, respectively.

ASSESSMENT OF AMF COLONIZATION

We assessed AMF colonization after 5, 17 and 29 months from the start of the experiment. Because the roots were obtained from soil cores, we were unable to distinguish roots of different plant species. Roots were separated and washed with water. All dead and damaged roots were discarded. All thin roots (< 2 mm without apparent suberin) that could be potentially colonized were cleared and stained following Grace & Stribley (1991). They were then mounted on semi-permanent slides in polyvinyl–lactic acid–glycerol; six slides per sample were prepared. The root endophyte quantification was made by the magnified intersection method (McGonigle *et al.* 1990) using a compound microscope (Kyowa optical, Model LSCB-VC-2B-L), magnification ×150. Eighty to 100 intersections per slide were scored depending on sample size (480–600 intersections per sample). Percentage of total, vesicle and arbuscule root colonization was assessed as the proportion of total root intersections that were colonized and was calculated as follows:

$$\text{Total colonization} = 100 \times (\text{Number of intersections with any AMF structure/Total number of intersections counted})$$

$$\text{Vesicle colonization} = 100 \times (\text{Number of intersections with AMF vesicles/Total number of intersections counted})$$

$$\text{Arbuscule colonization} = 100 \times (\text{Number of intersections with AMF arbuscules/Total number of intersections counted})$$

QUANTIFICATION OF AMF SPORES

We quantified AMF spore density 5, 17, 29, 36 and 39 months after the initial removals. Spores were extracted from 50 g of soil from each sample using a wet sieving and centrifugal flotation technique (Daniels & Skipper 1982). The extracted spores were observed under a light microscope and identified to species level using current morpho-taxonomic criteria (http://invam.caf.wvu.edu/). Only spores that appeared to be viable (based on external appearance and content) were counted. Voucher specimens were deposited in the Herbarium of the Museo Botánico de la Facultad de Ciencias Exactas, Físicas y Naturales de la Universidad Nacional de Córdoba (CORD).

Spore density (number of spores/100 g soil dry weight) was calculated by counting total spore number in each sample (each sample corresponding to one plot in the field). We also used spores to estimate Brillouin species diversity (HB), evenness indices (EB) 17, 29, 36 and 39 months after the first removals and the abundance of each functional group (i.e. grouping morphotaxa according to phylogenetic lineages that represent different functional groups in AMF, see Maherali & Klironomos 2007). We used Brillouin index because not all AMF species sporulate to the same degree, so not all of them have

an equal probability of detection in the soil sample (Magurran 1988). The indices were calculated according the following formulae:

$$\text{Evenness (EB)} = \text{HB}/\text{Hbmax}$$

where

$$\text{Diversity (HB)} = 1/\text{N} \times (\ln \text{N!} - \sum \ln \text{n}_\text{i}!)$$

where N = total number of spores in each sample (plot), n_i = number of individuals in species i and

$$\text{Hbmax} = 1/\text{N} \times \ln(\text{N!}/([\text{N/S}]!)^{\text{s}-\text{r}} \times \{([\text{N/S}]+1)!\}^{\text{r}})$$

where [N/S] = integer value of N/S, S = richness and r = N − S × [N/S]

ASSESSMENT OF NUTRIENT AVAILABILITY

We measured the accumulation of ammonium, nitrate and phosphate on ion exchange resins incubated in the soil to compare the relative availability of nitrogen (N) and P in the different treatments (Lajtha *et al.* 1999). Resin bags were made from nylon stocking material, which was soaked in 0.1 M HCl overnight before filling with ion exchange resins. Each bag contained 5 g FW of mixed-bed ion exchange resins (Amberlite IRN 150®, Philadelphia, PA, USA). Three resin bags were buried in each plot at approximately 5 cm depth from December 1999 to June 2000 and from December 2001 to April 2002. Resin bags were washed free of soil using distilled water, then extracted in 100 mL 2 M NaCl in 0.1 M HCl overnight. Extracted solutions were sent to IFEVA (University of Buenos Aires) where nutrients were measured with an Alpkem autoanalyser (O-I corporation College Station, TX, USA).

STATISTICAL ANALYSIS

AMF colonization of roots and density, richness, evenness and diversity of spores were analysed by a two-way ANOVA with removal treatment and block as main effects. The interaction term (removal treatment × block) was used as error term because there is only one replicate per treatment per block. For spore communities, we also searched for seasonal variation (see the Sampling section above) therefore we incorporated season in the model. For this, we used a repeated-measurement analysis through a univariate model for divided plots in randomized complete blocks (Crowder & Hand 1990). The model includes an effect of blocks, an effect of treatments (which are evaluated with the error corresponding to the mean square of 'block × treatment'), an effect of season and an effect of the season × treatment interaction. The effect of 'block × treatment' is interpreted as the experimental error. Fisher tests (LSD) were applied *a posteriori* to locate the differences among treatment means (Sokal & Rohlf 1998). When data showed heterogeneous variances that could not be corrected by log-transformation, they were rank-transformed, and two-way ANOVAS (same model as above) were run on the rank data (Zar 1999). In those cases, nonparametric analyses yielded the same conclusions as parametric ANOVAS run on the untransformed data, suggesting that it had sufficient power (Zar 1999). To detect general trends in morphotaxa composition of the mycorrhizal communities across plots and treatments, we submitted the AMF spores matrix to a principal component analysis (PCA). We then compared with ANOVA and Tukey's test the mean values of removal treatment scores along axis 1 and 2 of the PCA ordination. This way, we assessed if the overall AMF spore composition (summarized by axis 1 and axis 2 scores) differ among treatments. We also performed correlation analyses between colonization, spore variables and biomass removed at each sampling date and correlations between spore variables among seasons. All analyses were carried out with the INFOSTAT Statistical Package (Di Rienzo *et al.* 2002).

Results

AMF COLONIZATION

Five months after the initial removal operation, we found a significant reduction in total AMF colonization in all plots subjected to PFT removals and in the disturbed control plots, as compared with the undisturbed controls (Table 1, Fig. 1A). This effect later disappeared: 17 and 29 months after the initial removals no significant effect of any treatment was observed (Table 1, Fig. 1B,C). However, these responses were underlined by the contrasting behaviour of the different mycorrhizal structures. After 5 months, there was no significant change in the colonization by vesicles or arbuscules (Table 1, Fig. 2A). This lack of response was maintained through month 29 in the case of arbuscules, which never showed a detectable response and maintained low colonization levels (0.03–1.35%). In contrast, a significant increase in colonization by vesicles was observed in months 17 and 29 (Table 1). These increases corresponded to the disturbed control and the *no graminoid* treatment in month 17 (Fig. 2B), and only to *no graminoid* treatment in month 29 (Fig. 2C). No significant correlation between initial biomass removed and mycorrhizal colonization in each plot was observed.

AMF SPORE COMMUNITIES

We identified 13 distinct morphotaxa (see Table 2 and Tables S3 and S4). At the first sampling, 5 months after the initial removals, we only measured spore density and found no treatment effect (Table 1). After 17 months, all PFT removal treatments increased AMF spore evenness, but only in the case of the *no shrub* treatment was this increase significant relative to controls (Table 1, Fig. 3). After 29 months, we found that treatments did not significantly affect AMF spores in the soil.

No significant correlation was observed between biomass removed and AMF spores after 5 and 29 months. However, after 17 months we observed a significant negative correlation between biomass removed and spore density ($r = -0.4347$; $P = 0.03$) and a positive correlation with evenness ($r = 0.6219$; $P = 0.002$).

There was also no significant treatment effect on spore communities (density, diversity and evenness) even considering seasonal variation in the model. There was only a tendency towards higher diversity and evenness values in the *no shrub* and *no annual forb* treatments, mainly in summer (see Tables S2, S3 and S4). In addition, there was no significant difference in the abundance of AMF functional groups (i.e. Glomeraceae, Acaulosporaceae and Gigasporaceae) on each sampling date (data not shown). Nevertheless, when considering seasons in the model (repeated-measurement analysis), Gigasporaceae showed significantly higher spore abundance in

Table 1. Results of two-way ANOVA on the effects of treatments and blocks on arbuscular mycorrhizal fungi colonization and spores (d.f. = 5). Bold values indicate statistical significance at $P \leq 0.05$

	Source of variation											
	Block						Treatment					
	5 months		17 months		29 months		5 months		17 months		29 months	
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Total colonization	6.84	**< 0.01**	2.83	**0.04**	1.58	0.20	2.72	**0.04**	2.19	0.09	1.41	0.25
Vesicles	1.46	0.24	0.95	0.47	4.20	0.52	1.5	0.23	2.87	**0.04**	4.95	**< 0.01**
Arbuscules	1.27	0.31	0.06	0.99	0.65	0.66	0.14	0.98	0.44	0.82	0.34	0.88
Spore density	7.42	**< 0.01**	2.20	0.11	1.10	0.39	1.50	0.23	1.88	0.15	1.19	0.34
Spore diversity	–	–	1.05	0.42	0.20	0.96	–	–	2.04	0.12	0.83	0.54
Spore evenness	–	–	3.21	**0.03**	1.39	0.26	–	–	3.12	**0.04**	1.38	0.27

the *no shrub* treatment and the lowest values in *control* and *no perennial forb* treatments (see Table S5 and Fig. S1).

There was no clear difference in AM spore community composition among treatments (Fig. 4). Variation along PCA axes 1 was mainly explained by the spore density of *Glomus intraradices* in two *no graminoid*, one *disturbed control* and one *no shrub* plots, with plots under each treatment generally scattered on the ordination plane. Finally, the mean scores of removal treatments along axes 1 and 2 were not significantly different. There was only a trend of *no perennial forb* and *no graminoid* treatments to diverge towards opposite extremes of axis 1 (see Fig. 4 and Fig. S2).

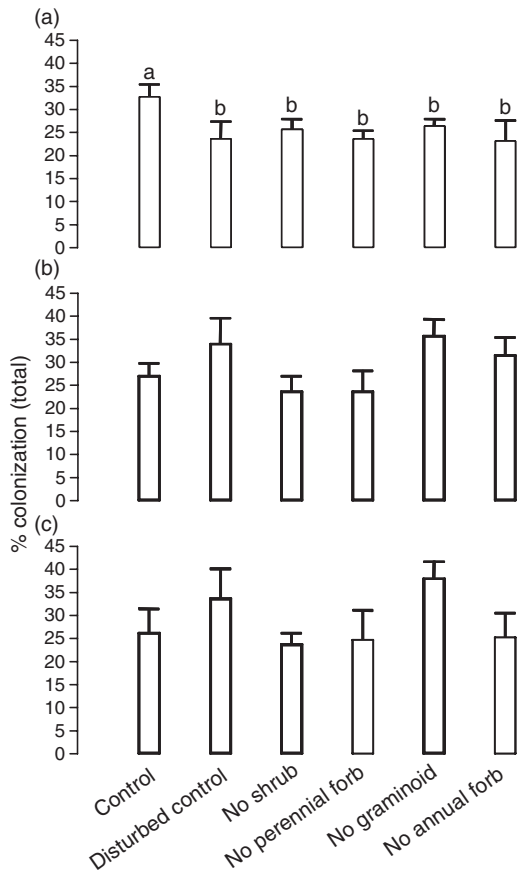

Fig. 1. Effects of different removal treatments (see text for full description) on arbuscular mycorrhizal fungi colonization. Bars represent total colonization (hypha + vesicles + arbuscules) 5 months (a), 17 months (b) and 29 months (c) after the initial removals. Error bars indicate +1 SE (n = 6 blocks). Bars with the same letters are not significantly different (Fisher's LSD test, $P < 0.05$).

NUTRIENT AVAILABILITY

In the period between 12 and 18 months after the initial removals, the *no annual forbs* treatment showed a significant increase in ammonia accumulated in the resin bags (Table 3,

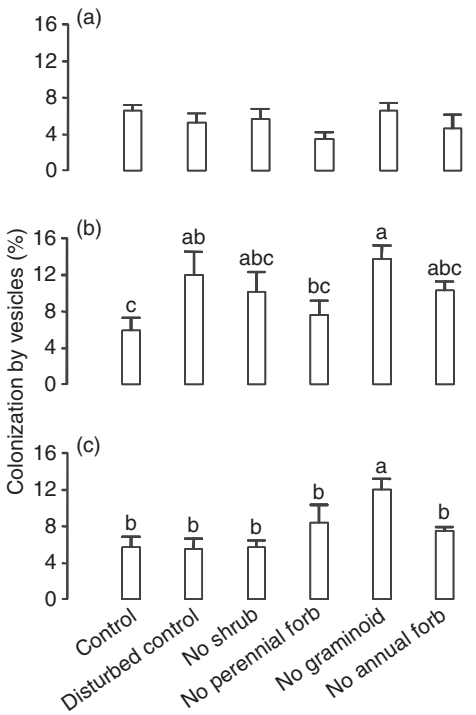

Fig. 2. Effects of different removal treatments (see text for full description) on colonization by arbuscular mycorrhizal fungi vesicles 5 months (a), 17 months (b) and 29 months (c) after the initial removals. Error bars indicate +1 SE (n = 6 blocks). Bars with the same letters are not significantly different (Fisher's LSD test, $P < 0.05$).

Table 2. Spore density (number 100 g^{-1} soil dry wt.) of different arbuscular mycorrhizal fungi morphotaxa, total spore density, diversity index and evenness index under different removal treatments after 29 months of the initial removal. *Glomus* sp. 1 and *Glomus* sp. 2 were only present after 36 and 39 months after initial removals (see Supporting information), therefore they are not listed in this table

Treatments	Control		Disturbed control		No shrubs		No perennial forbs		No graminoids		No annual forbs	
Arbuscular mycorrhizal fungi spores	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Glomus aggregatum	24.54	4.86	63.43	12.83	37.78	15.87	15.74	8.08	209.14	150.32	32.08	5.61
Glomus claroideum	0	0	3.46	3.46	1.55	0.98	1.54	1.54	17.38	9.83	7.86	2.98
Glomus intraradices	58.45	16.46	104.33	46.5	49.58	24.18	37.51	11	138.91	52.42	102.22	33.06
Glomus constrictum	16.92	5.81	10.67	4.17	9.14	2.54	11.76	4.01	21.23	6.09	15.72	7.59
Glomus sp. 3 (= *Sclerocystis* sp.)	0	0	0	0	0	0	0.81	0.81	0	0	0	0
Acaulospora mellea	0	0	0	0	0	0	0	0	0	0	0	0
Acaulospora laevis	8.03	5.12	16.07	9.87	1.95	1.27	2.8	2.39	8.14	3.38	11.92	6.01
Acaulospora scrobiculata	14.56	7.43	13.98	7.52	29.54	11.66	16.73	9.6	27.67	8.17	14.9	6.3
Entrophospora infrequens	2.74	2.74	8.3	3.09	13.51	11.91	1.47	1.08	6.18	2.79	1.03	1.03
Scutellospora biornata	1.2	1.2	0.39	0.39	1.52	1.52	0	0	3.7	3.7	1.85	1.85
Scutellospora sp.	8.16	3.87	3.77	2.39	9.19	2.65	2.76	1.18	8.71	3.1	9.61	3.5
Total spore number	132.24	20.02	217.88	20.02	153.85	57.5	89.49	27.87	441.07	187.57	196.32	45.6
Diversity index	1.37	0.06	1.17	0.06	1.49	0.1	1.39	0.08	1.38	0.11	1.35	0.09
Evenness index	0.57	0.03	0.49	0.03	0.62	0.04	0.58	0.04	0.57	0.04	0.56	0.04

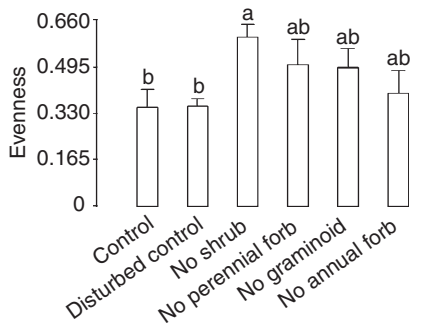

Fig. 3. Arbuscular mycorrhizal fungi spore evenness under different plant functional type removal treatments after 17 months. Error bars indicate +1 SE (n = 6 blocks). Bars with the same letters are not significantly different (Test LSD Fisher test, $P < 0.05$).

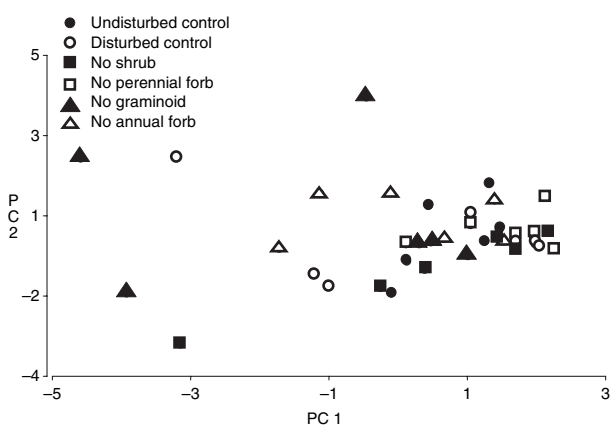

Fig. 4. Principal component analysis (PCA) of plots under different removal treatments, on the basis of arbuscular mycorrhizal fungi (AMF) spore composition after 29 months of initial removals. Eigenvector scores of AMF morphotaxa in the two PCA axes are shown in Table S6.

Fig. 5A). Later, in the period between 24 and 28 months after the initial removals, the effect of *no annual forbs* disappeared and the *no shrubs* treatment showed a significant decrease of nitrate (Table 3, Fig. 5B).

Discussion

INITIAL EFFECT OF REMOVAL TREATMENTS

The negative effects of the disturbed control and all removal treatments on total mycorrhizal colonization 5 months after the start of the experiment suggest that the immediate effects of the treatments could be attributed to the physical disturbance associated with the initial removal operation. This is not surprising since this initial effect is one of the main artefacts of the field removal approach (Díaz *et al.* 2003). In the specific case of AMF, several authors have previously reported the negative relationship between physical disturbance and colonization (Jasper, Abbott & Robson 1989; An *et al.* 1993; Merryweather & Fitter 1998). This effect has been attributed to the fact that physical disturbance of the soil is often followed by increased mineralization of soil organic matter, which results in nutrient flushes that in turn can reduce mycorrhizal colonization (Smith & Read 2008). In this experiment, no effect of disturbance on nutrient availability (as determined by the use of ion exchange resin bags) was observed. Therefore, our results suggest that the soil and rhizosphere manipulations associated with the removal and disturbed control treatments may have directly disrupted the extraradical mycelium (Evans & Miller 1988; Read & Birch 1988). This in turn might have negatively affected intraradical AMF colonization. These results highlight the importance of including in removal experiments a disturbed control treatment that accounts for the initial side effects attributed to physical and biotic alteration of the soil caused by the act of removal (Díaz *et al.* 2003).

Table 3. Results of two-way ANOVA on the effects of treatments and blocks on nutrient availability, as measured by ion accumulation on resin bags (d.f. = 5). Bold values indicate statistical significance at $P \leq 0.05$

	Source of variation							
	Block				Treatment			
	1999–2000		2001–2002		1999–2000		2001–2002	
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Ammonia	3.044	**0.028**	5.476	**0.002**	3.429	**0.017**	1.560	0.208
Nitrate*	2.076	0.102	6.139	**0.001**	1.492	0.228	4.196	**0.007**
Phosphate	2.195	0.087	3.182	**0.023**	0.257	0.932	0.693	0.633

*Data were rank-transformed because their variance was not homogeneous and could not be made homogeneous by log-transformation.

RESILIENCE OF AMF COLONIZATION IN THE FACE OF DISTURBANCE

Seventeen months after the initial removals, effects of treatments or initial disturbance on AMF colonization were no longer detectable, and this was still the case after 29 months. This suggests that AMF communities are highly resilient in the face of disturbances of the canopy and the soil physical structure such as those applied in our experiment. Similarly, rapid recovery of AMF colonization after disturbance has been previously observed (Merryweather & Fitter 1998), suggesting that short-term experiments on mycorrhizal community structure should be interpreted with caution. Although these results may overlook changes in mycorrhizal composition in roots, it has been shown that changes in AMF composition in roots can be also related with changes in the percentage of colonization because AM fungi vary in the amount of root they colonize, either alone or in mixtures (Jansa, Smith & Smith 2008).

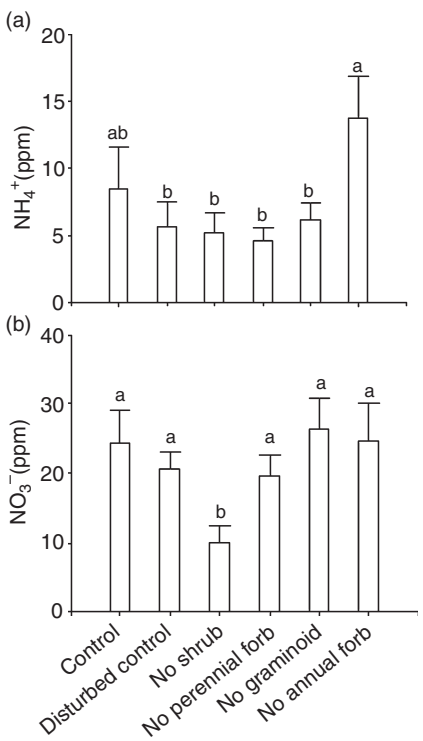

Fig. 5. Effects of different removal treatments (see text for full description) on the accumulation of ammonium (a) and nitrate (b) by resins in period between 12–18 and 24–28 months after the initial removals, respectively. Error bars indicate +1 SE (n = 6 blocks). Bars with the same letters are not significantly different (Fisher's LSD test, $P < 0.05$).

RESILIENCE OF AMF COMMUNITIES UNDER DIFFERENT PFT TREATMENTS

Once they had recovered from the disturbance associated with the initial removal operation, no difference in AMF colonization was observed under different PFT manipulations, even under those that involved dramatic changes in above-ground biomass and alteration of soil nutrient availability in the understorey. The fact that AMF colonization indeed decreased in the first months after the initiation of the experiment and then recovered rules out the possibility that this lack of response could be attributed to resistance or inertia of AMF communities, or to inability of our measurement protocols to detect differences. It could be argued that the similar AMF colonization among different PFT treatments could be due to the capacity of the remaining PFTs to compensate for the lost biomass of the removed PFTs. However, in the same experimental setup, Gurvich (2005) found no significant above-ground biomass compensation of the removed biomass by remaining PFTs. Although it has been shown that changes in AMF composition in roots are also related with changes in the percentage of colonization because AM fungi vary in the amount of root they colonize (Jansa, Smith & Smith 2008), we cannot exclude the possibility of the occurrence of changes in mycorrhizal composition and functioning without changes in the overall rate of colonization.

Despite the general assumption that AMF are generalists, their effects on plant species vary according to different plant–fungal combinations (e.g. van der Heijden *et al.* 1998; Scheublin, van Logtestijn & van der Heijden 2007). In addition, differential sporulation of AMF species grown with different host plants is well documented (Johnson, Tilman & Wedin 1992; Bever *et al.* 1996; Eom, Hartnett & Wilson 2000;

Lovelock, Andersen & Morton 2003; Lovelock & Ewel 2005), suggesting some level of specificity in this association. Nevertheless, Lovelock, Andersen & Morton (2003) found that in tropical forest, although there was a species effect on AMF spore communities, no effect of plant life history groupings was observed. Although we found some seasonal changes in Gigasporaceae spore abundance, the lack of a consistent effect of PFTs on the most abundant AMF functional groups (Glomeraceae and Acaulosporaceae), as well as the other spore variables observed in our experiment, are in line with those observations and suggest that AMF-community differences between host plants might disappear beyond the scale of host species. In other words, despite some level of host specificity or preference for some AMF functional group or taxa, the removal of certain hosts or groups of hosts (such as PFTs) seems unlikely to lead to local extinction of AMF species or to changes in the relative abundance of AMF functional groups. This is probably because in natural communities the availability of compatible hosts is highly unlikely to reach low enough levels to exclude AMF species from root colonization, except for great disturbances or drastic changes in native communities (e.g. from natural forest to crops; Helgason *et al.* 1998). The possibility exists that the behaviour of AMF in the face of plant removals could have been to lay down resting spores as an immediate response to the manipulations, thus explaining the lack of differences between treatments. However, if the spores indeed responded in this way, after 3 years in the soil the resting AMF spores would have germinated to reach a host or would have perished as a result of the action of the complex natural soil food web, including fungivorous invertebrates such a nematodes and collembola. In fact, some transient effects were observed: after 17 months spore evenness was significantly enhanced by all removal treatments, especially *no shrubs* treatment. This effect seems to be related to the biomass removed since there was a significant correlation between this variable and spore evenness. Whatever the mechanisms behind these effects, the AMF spore community showed resilience to them, since the differences were absent after a relatively short period. Moreover, no AM morphotaxon was absent in any treatment. This agrees with a recent study that imposed a different kind of disturbance to the soil fungal community (application of the fungicide benomyl), and found that no AM DNA-based taxa were eliminated under this treatment in comparison with the control (Helgason *et al.* 2007). Moreover, in natural communities AMF spore diversity did not consistently change during conversion of tropical forest to grassland and other types of communities (Johnson & Wedin 1997; Picone 2000; Violi *et al.* 2008). In grassland microcosms, Johnson *et al.* (2004) found no effect of plant biomass on AMF communities. In a grassland removal experiment, Wardle *et al.* (1999) found that after 3 years decomposer soil biota were not strongly affected by removal of several PFTs (C_4 grasses, C_3 annual grasses, C_3 perennial grasses, clovers and dicotyledonous weeds), and showed no significant relationship with plant biomass or productivity. Our experiment suggests that this is also true in the case of plant communities consisting of more contrasting PFTs (i.e. herbaceous and woody species).

Our findings contrast with those observed in similar field removal experiments for ericoid mycorrhizas and ectomycorrhizas. In the arctic tundra of Alaska, the removal of the shrub *Betula nana* decreased ericoid mycorrhizal colonization, and the removal of *Ledum palustre* decreased ectomycorrhizal colonization and affected ectomycorrhizal morphotype composition (Urcelay *et al.* 2003), while in northern Arizona, the removal of AMF-infected shrubs increased ectomycorrhizal colonization even though it did not affect composition (McHugh & Gehring 2006). These results suggest that ericoid and ectomycorrhizal fungi (Phyla Ascomycota and Basidiomycota) are more responsive to changes in vegetation than AMF (Glomeromycota). This is consistent with the widely known fact that Ascomycota and Basidiomycota are considerably more host-specific than Glomeromycota (Kottke *et al.* 2008; Smith & Read 2008).

In our experiment, despite the lack of differences in total mycorrhizal colonization in the longer term, percentage of colonization by vesicles was not affected by initial disturbance but was consistently enhanced by removal of graminoids after 17 and 29 months of removal. Most graminoids, and the dominant *Stipa eryostachia* in particular, showed a low percentage of vesicle colonization (Urcelay & Battistella 2007). Therefore, the higher proportion of roots of PFTs other than graminoids present in the *no graminoid* plots might account for the higher vesicle colonization observed in this treatment. It is known that AMF species belonging to the Gigasporaceae do not form vesicles, therefore it raises the possibility that the observed reduction in vesicle colonization could be a consequence of a reduction of Gigasporaceae in the *no graminoid* plots. When we examined this possibility, Gigasporaceae showed significantly higher values in *no graminoid*, but also in *no shrub* and to a lesser degree *disturbed control* in comparison with *control* and *no perennial forb*.

It has been recently shown in synthetic community experiments that plant effects on soil chemical properties and microbial communities can be highly idiosyncratic. For example, plant composition affects chemical properties but not microbial communities in sandy-soil communities in the Netherlands, but shows the opposite effects in chalk soil communities in the UK (Bezemer *et al.* 2006). In our experiment, no effect of disturbance or treatments on phosphate availability was observed, but removal of annual forbs promoted an increase of ammonia in the shorter term while removal of shrubs promoted lower nitrate availability (Fig. 5B) and soil temperature (Gurvich 2005) in the longer term. This decrease of nitrate availability could be attributed to the absence of the nitrogen-fixing legume *Acacia caven*, which is the most abundant shrub (Table S1). The lack of major treatment effects on AMF communities suggests that no nutrient- or temperature-mediated effect of PFTs removals existed. Although soil properties such as nutrients (Johnson *et al.* 2003; Smith & Read 2008) and temperature (Heinemeyer *et al.* 2003; Staddon *et al.* 2003) are known to affect AMF colonization and community structure in synthetic experiments, the magnitude of the changes observed in our field experiment might have not been strong enough to affect the fungal variables measured.

POSSIBLE ASYMMETRY IN THE MUTUAL EFFECTS OF AMF AND PLANTS

Overall, and after applying removal treatments on a native shrubland during approximately 3 years, our results do not provide consistent evidence for direct or indirect effects of different PFTs on AMF colonization or spore community parameters. The removal of PFTs with more conservative trait syndromes (e.g. shrubs; Díaz & Cabido 1997) did not affect mycorrhizal parameters in a way consistently different from that of more acquisitive syndromes (e.g. fast-growing annual forbs). This is inconsistent with the hypothesis of differential effects of plants with different resource-use syndromes (conservative vs. acquisitive; Diaz *et al*. 2004) on soil microbial communities. On the other hand, and except for some transient effects, the effect of different removal treatments was not directly proportional to the biomass removed, which is inconsistent with the biomass-ratio hypothesis. These results add evidence to the suggestion that belowground organisms are generally less responsive and/or respond more slowly to changes in vegetation than aboveground organisms (Wardle 2002, 2006). This could be particularly true for those organisms that are not highly host- or substrate-specific, such as AMF. Whether the resilience showed by mycorrhizal colonization and spore composition in the soil relates to resilience of AMF composition in roots or extraradical mycelium remains unanswered. This is because there may be no direct relationship between spore diversity in the soil and AMF taxa colonizing roots (Sanders 2004).

Because root mycorrhizal colonization is a dynamic variable that mainly relates to nutrient acquisition and plant productivity on the one hand, and mycorrhizal spores relate to seedling establishment after disturbance on the other hand (van der Heijden & Scheublin 2007), the ecological interpretation of the results reported here should be framed within these processes. Considering the facts that (i) AMF range from mutualistic to parasitic (Johnson, Graham & Smith 1997; Smith & Read 2008); (ii) they differentially affect the growth of plant species (van der Heijden *et al*. 1998; van der Heijden, Wiemken & Sanders 2003; Maherali & Klironomos 2007; Scheublin, van Logtestijn & van der Heijden 2007) or groups (e.g. forbs, C_4 grasses and C_3 grasses, Hetrick, Kitt & Wilson 1988; Wilson & Hartnett 1998); (iii) their removal has been shown to affects plant community structure in the field at shorter time scales than the one considered here (Gange, Brown & Sinclair 1993; Newsham *et al*. 1995; Hartnett & Wilson 1999; O′Connor, Smith & Smith 2002) and (iv) functional groups of AMF can have different effects on plant growth (Maherali & Klironomos 2007), our findings suggest that the hypothesis of a possible asymmetry in the plant–AMF interactions at the community level deserves explicit testing. This reinforces the need to carry out studies on the feedback effects between plants and AMF in natural communities in order to understand above- and belowground interactions caused by changes in land use and vegetation composition.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of the most abundant species and plant functional types (PFTs) in the study site at the beginning of the removal experiment.

Table S2. Spore communities under different removal treatments, blocks, seasons and season × treatment interaction as main effects.

Table S3. Mean arbuscular mycorrhizal fungi spore density (number per 100 g soil dry weight), total density, diversity index and evenness index among treatments after 36 months of initial removals.

Table S4. Mean arbuscular mycorrhizal fungi spore density (number per 100 g soil dry weight), total density, diversity index and evenness index among treatments after 39 months of initial removals.

Table S5. Results of a repeated measurement analysis of arbuscular mycorrhizal fungi functional groups through a univariate model for divided plots in randomized complete block with effect of block, treatment, season and season × treatment interaction (data were rank transformed).

Table S6. Eigenvector scores of arbuscular mycorrhizal fungi morphotaxa in two main PCA.

Figure S1. Gigasporaceae spore abundance under different plant functional type removal treatments along seasons (see Materials and methods). Error bars indicate +1 SE ($n = 6$ blocks). Bars with the same letters are not significantly different (Test LSD Fisher test, $P < 0.05$).

Figure S2. Box plots illustrate the distribution of treatments along PCA axis 1. Score means distribution were not significantly different between treatments ($F = 1.9717$, $P = 0.1180$).

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