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Research

Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms

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Summary

• The diversity of arbuscular mycorrhizal (AM) fungi was investigated in an unfertilized limestone grassland soil supporting different synthesized vascular plant assemblages that had developed for 3 yr.

• The experimental treatments comprised: bare soil; monocultures of the nonmycotrophic sedge *Carex flacca*; monocultures of the mycotrophic grass *Festuca ovina*; and a species-rich mixture of four forbs, four grasses and four sedges. The diversity of AM fungi was analysed in roots of *Plantago lanceolata* bioassay seedlings using terminal-restriction fragment length polymorphism (T-RFLP). The extent of AM colonization, shoot biomass and nitrogen and phosphorus concentrations were also measured.

• The AM diversity was affected significantly by the floristic composition of the microcosms and shoot phosphorus concentration was positively correlated with AM diversity. The diversity of AM fungi in *P. lanceolata* decreased in the order: bare soil > *C. flacca* > 12 species > *F. ovina*.

• The unexpectedly high diversity in the bare soil and sedge monoculture likely reflects differences in the modes of colonization and sources of inoculum in these treatments compared with the assemblages containing established AM-compatible plants.

Key words: terminal restriction fragment length polymorphism (T-RFLP), arbuscular mycorrhiza (AM), plant functional type; diversity; shoot phosphorus, shoot nitrogen.

Introduction

Arbuscular mycorrhizal (AM) fungi link above- and belowground ecosystem functioning and, through their contribution to nutrient cycling and plant productivity, are among the most important soil organisms (Smith & Read, 1997). In addition, increasing recognition has been given to the pivotal role that AM play in plant community ecology through their beneficial effects both on plant diversity and plant productivity (Grime *et al.*, 1987; Klironomos & Hart, 2002). There is some evidence that these functions may be enhanced with increasing diversity of AM fungi (van der Heijden *et al.*, 1998a,b; Klironomos *et al.*, 2000; O'Connor *et al.*, 2002).

While the effects of AM diversity on plant community diversity have recently attracted considerable attention, the reciprocal question as to the effect of plant diversity on AM diversity and mycorrhizal community functioning has been surprisingly neglected. This may be a consequence of the established view that because only about 150 species of AM fungi have been described (Walker & Trappe, 1993), and the

symbiosis is found in 90% of plant families (Clapp *et al.*, 2002), these fungi must have very low host specificity (Vanderplank, 1978). A logical consequence of this view would be that AM communities are little influenced by plant species composition.

These conclusions have recently been challenged on several fronts. Important insights into AM fungal community composition have been attained by targeting the small subunit (SSU) rRNA gene as a molecular marker, which avoids the need for culturing and spore identification. The development of primers to identify AM fungi colonizing plant roots (Helgason *et al.*, 1998; Vandenkoornhuyse *et al.*, 2002a) has made it possible to study the diversity of AM fungi with a greater degree of certainty and precision than was hitherto possible. These studies (Helgason *et al.*, 1998, 1999; Daniell *et al.*, 2001; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002a,b) reveal a greater diversity of AM fungi in roots than could be recognized by morphometric studies. They have also resulted in the discovery of previously unrecorded species (Helgason *et al.*, 1998, 1999; Daniell *et al.*, 2001; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002a,b, 2003) and evidence of hitherto hidden host specificity (Bidartondo *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002b, 2003). Furthermore, such studies have revealed that both the composition of AM fungal communities in roots (Helgason *et al.*, 2002; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002b, 2003), and the production of their spores (Bever, 2002) can be influenced by the host plants.

At present, little is known about the control of diversity of AM fungal communities and,, given the increasing importance attached to mycorrhizal fungal diversity for maintenance of ecosystem functioning (van der Heijden *et al.*, 2003), a better understanding of the causes of AM diversity (and its loss) is clearly required. In particular, both from an agronomic and ecosystem perspective, the effects of plant species composition and diversity on AM diversity is of considerable interest.

Here, we test the hypothesis that plant functional type and diversity influences AM fungal diversity. In particular, we were interested to test the effects on AM diversity of monocultures of host plants (*Festuca ovina*) and nonhost plants (*Carex flacca*) against bare soil and a 12-species mixture of mainly mycorrhiza-compatible plants. We hypothesized that mycorrhizal diversity would be reduced in the bare soil and monocultures of *C. flacca* because they do not support extensive AM colonization, but that it would be greater in microcosms containing 12 plant species than in monocultures of *Festuca* because the more diverse plant community could be expected to support more host-specific fungi.

We tested these hypotheses using bioassay seedlings of *Plantago lanceolata* introduced into microcosms comprising 3-yr-old grassland assemblages. The microcosms are part of a larger, long-term experiment investigating the effects of vascular plant identity and diversity on ecosystem functioning (Grime, 2001a,b) and were assembled from established plants removed from a limestone grassland and sown into coarsely sieved soil from the field. The diversity of AM fungi was determined in roots of the bioassay seedlings by terminal-restriction fragment length polymorphism (T-RFLP). This is a highly sensitive and reproducible molecular tool, pioneered by Liu *et al.*, 1997), that enables comparisons of community structure without the need of cloning and sequencing (Clement *et al.*, 1998; Osborn *et al.*, 2000; Zhou & Hogetsu, 2002). The functional attributes of AM fungal communities were inferred from measurements of plant biomass production and shoot nitrogen (N) and phosphorus (P) concentrations of *P. lanceolata* bioassay seedlings. We accounted for potential confounding factors (e.g. differences in the shoot biomass of the established microcosm assemblages) by using analysis of covariance in a general linear model.

Materials and Methods

Establishment of grassland microcosms

In 1997, 3 yr before commencement of the experiments described here, rendzina soil was removed from the surface 20 mm of a species-rich undisturbed limestone grassland at the Buxton Climate Change Impacts Laboratory in Derbyshire, UK (National Grid Reference (NGR): SK 055706). The soil was coarsely sieved (10 mm) to remove stones, thoroughly mixed and packed into free-draining 600 × 600 mm wide, 150 mm deep acrylic boxes at the University of Sheffield Experimental Gardens, UK. Shortly afterwards, into each box, except for a set of bare-soil controls, 192 mature plants matched in size were transplanted into the microcosms in a 14 × 14 grid (each corner remained empty). The plants originated from an ancient species-rich limestone grassland at the Cressbrookdale National Nature Reserve, UK (NGR: SK173735), and all were long-lived, slow-growing species coexisting in the vegetation (Booth & Grime (2003) for full details). Three vegetated treatments were produced: monocultures of *C. flacca* and of *F. ovina* and a 12-species mixture. These assemblages comprised plants representative of three broad taxonomic functional types common to limestone grasslands; sedges, grasses, and forbs. Extensive colonization by mycorrhizal fungi has been observed in the roots of the latter two groups in the same grassland (Read *et al.*, 1976) and the microcosm communities containing these plants would include their mycorrhizal fungal partners. The 12 species comprised the sedges *Carex caryophyllea* Latour., *Carex flacca* L., *Carex pulicaris* L., *Carex panicea* L., the grasses *Briza media* L., *Festuca ovina* L., *Helictotrichon pratense* (L.) Pilger, *Koeleria macrantha*; and the forbs *Campanula rotundifolia* L., *Leontodon hispidus* L., *Succisa pratensis* Moench, *Viola riviniana* Rchb. The positions of the plants in the mixed species microcosms were randomized, and each microcosm treatment was replicated four times in a randomized block (16 microcosm treatments in total). The plant species composition in the microcosms was maintained by regular weeding, and the short turf maintained by annual

clipping to a height of 25 mm. During the 3-yr period the plant assemblages became well established (Grime, 2001b).

Shoot biomass, mycorrhizal colonization and soil properties

The main plant and soil characteristics of the microcosms were determined to establish whether there were factors, in addition to floristic differences, that might affect mycorrhizal colonization and growth of the bioassay seedlings. Estimates of above-ground biomass to soil level of the established assemblages in each of the microcosms were made in August 2000 by removing vegetation within three replicate 60 mm diameter circles located at random from each microcosm followed by oven-drying and weighing.

The extent of root colonization by AM fungi in the *C. flacca*, *F. ovina* and the 12-species plant assemblages was determined from roots sampled in cores taken from each of the replicate microcosms after staining washed samples of roots with Trypan blue in lactoglycerol (Giovannetti & Mosse, 1980).

We anticipated that the differences in composition and productivity of the plant assemblages accumulated over 3 yr might have resulted in significant alteration in soil properties in the different microcosms, especially in the amount of soil carbon and macronutrients nutrients present. Total soil nitrogen (N) and carbon (C) contents were determined on finely ground oven-dried bulk samples by isotope ratio mass spectrometry (ANCA-GSL preparation module connected to a 20–20 stable isotope ratio mass spectrometer; PDZ Europa, Middlewich, UK). Soil phosphorus (P) concentrations were determined using the molybdenum blue method (John, 1970) after digesting 100 mg d. wt soil in 1 ml salicylic/sulphuric acid.

Bioassay of plant effects on AM diversity (Experiment 1)

In July 2000, 5-d-old-seedlings of *P. lanceolata* that had been germinated in a growth room (20°C, 18 h light/15°C, 6 h dark) on autoclaved calcareous sand (obtained from Anglesey, north Wales, UK) were transplanted into three of the four replicate microcosm treatments ($n = 3$ for each treatment). Only three replicate microcosms were used because of restrictions on the number of samples that could be processed. *Plantago lanceolata* L. was chosen as the bioassay test species because it is commonly found in limestone grasslands and it is highly mycorrhiza-responsive. It has also been shown to host a very broad range of AM fungi, including representatives of five major genera: *Glomus*, *Acaulospora*, *Entrophospora*, *Scutellospora* and *Gigaspora* (Klironomos & Hart, 2002). The seedlings were harvested after 12 wk of growth.

Assessment of AM diversity within roots of *P. lanceolata* seedlings by T-RFLP Roots of *P. lanceolata* seedlings were taken from each microcosm, thoroughly washed and frozen separately. Total DNA was extracted from the roots of each seedling according to Edwards *et al.* (1997) followed by an additional purification step using the Concert Rapid Purification kit (Life Technologies, Invitrogen Ltd, Paisley, UK).

Because of the early radiation of the Glomeromycota (Schüßler *et al.*, 2001) the small subunit (SSU) rRNA gene provides sufficient variation to discriminate AM fungal isolates below the species level (Vandenkoornhuyse & Leyval, 1998). Therefore part of the SSU rRNA gene was amplified (550 bp) using a universal eukaryotic primer NS31 (Simon *et al.*, 1992), and an AM fungal-specific primer AM1 (Helgason *et al.*, 1998). The polymerase chain reaction (PCR) amplifications were allowed to run in a PTC100 machine (MJ Research Inc., Waltham, MA, USA). The PCR amplification products were synthesized and end-labelled with fluorescein dyes with a mixture containing 10 pmol of NS31-HEX, 10 pmol of AM1-6-FAM, 0.725 units of proof-reading *Pfu* DNA-polymerase (Promega UK Ltd., Southampton, UK), in full-strength manufacturer's reaction buffer and 0.2 mM of each dNTP (Gibco BRL, Crewe, UK; Life Technologies, Invitrogen Ltd). The cycling regime used was the same as described by Helgason *et al.* (1999). Labelled PCR products were purified as described for total root DNA to remove excess labelled primers. Purified PCR products were eluted in one volume of Tris/EDTA buffer.

The level of resolution of the T-RFLP method is greatly improved by using more than one restriction enzyme to generate polymorphism for the terminal restriction fragments (T-RFs) at both labelled ends. The purified 18S rDNAs were therefore digested separately with two different restriction enzymes (*Hin*fI and *Hsp*92II; Promega). Each digestion was performed in full-strength *Hin*fI or *Hsp*92II buffer, 2 units of enzyme (*Hin*fI or *Hsp*92II) and 15 µl of the purified, labelled amplicons, and incubated for 4 h at 37°C. The choice of the restriction enzymes was based on the frequency and size distribution of T-RFs. On the basis of the known and available sequences of AM fungi, the enzymes selected (*Hin*fI and *Hsp*92II) showed the best polymorphism of cleavage sites at the extremities of the amplified DNA fragment.

The digestion products were precipitated with 2 µl of a 2 M acetate buffer (pH 4.6) and 50 µl of absolute ethanol. The pellet was washed in 70% ethanol and dried at 42°C for 20 min Pellets were suspended in 3 µl of formamide/ethylenediaminetetraacetic acid (EDTA) (50 mM, pH 8.0) to which 0.5 µl of loading buffer and 0.5 µl of internal lane size standard GENESCAN-400HD-ROX (Perkin Elmer, Wellesley, MA, USA) were added. Samples were loaded on a standard sequencing gel after being denatured for 3 min at 90°C and were run at 52°C on an ABI377 automated sequencer (Perkin Elmer). The T-RF sizing and diversity signatures were performed using the GeneScan software (Applied Biosystems, Foster City, USA). The internal line standard chosen (GENESCAN-400HD-ROX) allows an accurate sizing of fragments between 45 and 450 bp long. All bands smaller or greater than this size range were therefore excluded for all the data analyses.

Reproducibility of AM fungal T-RF patterns was confirmed using two internal replicates (i.e. each terminal-restriction fragment length polymorphism (T-RFLP) analysis duplicated on separated sequencing slab gels). Only the T-RF length distributions were taken into account for data analyses because the peak heights of the T-RFs varied between replicates. Thus, for all data analyses presented only species richness is analysed (presence/absence of a T-RF) while species evenness (T-RF peak height or surface area) is not considered.

Analysis of T-RFLP data to determine AM diversity and community responses to plant assemblages The AM fungal communities associated with *P. lanceolata* roots were characterized by the number of peaks in fluorescence intensity of T-RFs of different basepair lengths. Pairwise comparisons among T-RF patterns of root-associated fungi were undertaken within and between microcosms. To estimate the level of homology among T-RF patterns, the average similarity coefficient (S) was computed using the relation

$$S = 2N_{ab}/(N_a + N_b)$$

(N_a and N_b are the number of T-RFs observed in fungal community A and B; while N_{ab} is the number of common T-RFs observed in A and B; Lynch, 1990). The average similarity coefficient between the mycorrhizal diversity signatures (T-RF patterns) in *P. lanceolata* grown in the different microcosms was calculated to determine effects of plant assemblage composition on AM diversity and species composition.

Genetic diversity (hereafter meaning species richness) was estimated using Shannon's information measure, H_0, calculated by the formula:

$$H_0 = -\sum p_i \ln p_i$$

(p_i is the frequency of a given T-RF fragment). It is possible to calculate H_0 for two levels: the diversity of AM fungi colonizing *P. lanceolata* (H_{tot}) (i.e. all the T-RF patterns are taken into account, $n = 12$) and the average diversity within microcosm treatments (H_{mic}) (i.e. for each of the four microcosm treatments, $n = 3$). Hence, the proportion of diversity within populations (i.e. microcosm treatment) can be estimated as H_{mic}/H_{tot}, while the proportion of diversity between microcosm treatments can be estimated as $(H_{tot} - H_{mic})/H_{tot}$. The proportion of diversity within and between microcosms was computed separately for the two restriction enzymes used to generate the T-RF patterns.

The fungal community structure and pattern of diversity was assessed by calculating a dendrogram (Losos, 1996). For each environmental sample, a given T-RF (or a SSU rRNA gene type) was scored as either 0 for absence or 1 for presence. The most parsimonious tree was found using the 'Branch and Bound' algorithm implemented in PAUP 3.1.1 (Swofford, 1993). The bootstrap values, estimated from 500 replicates using the 'Branch and Bound' procedure, reflect the percentage identical topology of the branches.

Biomass, nitrogen and phosphorus content of bioassay seedlings At harvest, the *P. lanceolata* shoots were excised, oven dried (80°C for 48 h), weighed and digested in 1 ml of a salicylic/sulphuric acid-lithium sulphate mix for 5 h at 350°C (Bremner & Mulvaney, 1982), diluted to 10 ml and analysed for total N (Scheiner, 1976) and P (John, 1970).

Assessment of the amount of AM colonization in *P. lanceolata* seedlings (Experiment 2)

A second bioassay experiment was undertaken in September 2001 in order to determine the effects of the plant assemblages on the amount of AM colonization. Sixteen seedlings of *P. lanceolata* were germinated as described earlier and transplanted into each of the four microcosm treatments. The bioassay seedlings were removed after 21 d of growth and the extent of colonization in their roots determined as described for the bulk root samples. In addition, the percentage of root length occupied by arbuscules and vesicles was recorded.

Statistical analyses

In order to determine whether the effects of the plant assemblages on AM diversity in the bioassay seedlings was related to differences in productivity of the established assemblages we ran a series of analyses of covariance (ANCOVA). The data from AM diversity analyses were combined with the shoot weights of the bioassay seedlings, the microcosm plant assemblage biomass data and the microcosm treatments to determine which of these factors significantly affected AM diversity. Similarly, ANCOVA was run to determine the extent to which growth and nutrient status of the bioassay seedlings could be related to AM diversity and to potentially confounding effects of the seedling biomass and established plant assemblage shoot biomass. Total N- and P-values for whole shoots were not used because of autocorrelation with biomass. The ANCOVAs were run in general linear models (with a diagonal covariance structure and restricted maximum likelihood (REML) estimation method) using SAS for Windows version 8 (SAS Institute Inc., Cary, NC, USA). A backward, stepwise procedure was conducted such that all factors were initially included in the model, but the least significant factors were sequentially removed from the model.

Elsewhere, differences between means were analysed by ANOVA followed by the Tukey honestly significant difference multiple comparison test. Percentage data were arcsine transformed. *P. lanceolata* shoot dry weight data were normalized by $\log_{10}$ transformation and the means presented are back-transformed values.

Results

Above-ground biomass, mycorrhizal colonization and soil properties

As intended, the biomass of shoots of established vascular plants varied significantly, ranging from 0 in the bare soil microcosms, and from 20 to 56 g d. wt m^{-2} in those that were vegetated. The *C. flacca* monocultures produced the smallest shoot biomass and the greater value was for the 12-species mixture, with the *F. ovina* monoculture lying between these (Table 1).

Analysis of bulk root samples from the *C. flacca*, *F. ovina* and 12-species microcosms showed that the percentage root length colonized by AM fungi ranged from 4% in the sedge and increased significantly ($P < 0.001$) to 70% in the highly mycorrhiza-receptive grass, and 60% in the mixed species communities (Fig. 1).

The soil properties were also affected by the microcosm treatments, with the bare soil treatment being the most profoundly different from the others. Samples from this treatment had a significantly lower pH than the 12-species communities, significantly lower carbon content than all the vegetated microcosms and lower total nitrogen content than the *F. ovina* and 12-species assemblages (Table 1). The C : N ratio was also significantly reduced in the bare soil treatment compared with those with vegetation. These differences reflect both the lack of input of new carbon over the 3-yr period into the bare soil microcosms and the almost 10-fold greater rate of leaching of nitrate measured in this treatment (see Grime, 2001a). There were no detectable differences in total soil P concentration between the microcosms. The only significant difference in soil properties detected between the vegetated treatments was a reduction in total soil N from 6.8 mg g^{-1} with 12 species assemblages to 6.0 mg/g with *C. flacca* monocultures (Table 1).

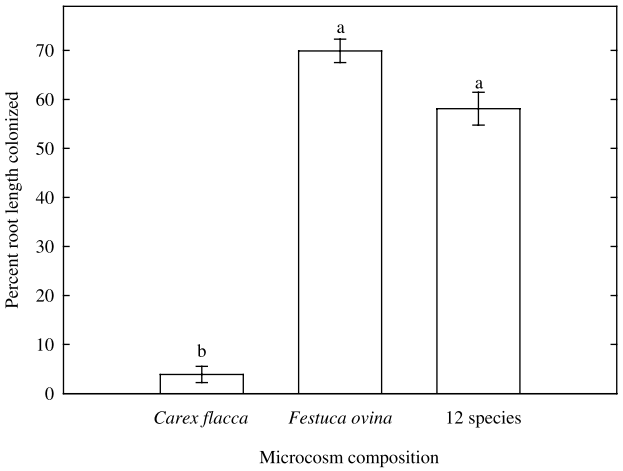

Fig. 1 Percentage root length colonized by arbuscular mycorrhizal (AM) fungi of bulk samples removed from grassland microcosms of *Carex flacca* and *Festuca ovina* monocultures and a mixture of 12 species (± SE; $n = 4$). Bars with the same letter are not significantly different ($P > 0.001$).

Effect of plant assemblages on AM diversity in *P. lanceolata* bioassay seedlings analysed by T-RFLP (Experiment 1)

A total of 63 T-RFs were found in the size range 45–450 bp and of these, 28 were found only once, and no T-RF was found across all 12 plants. Representative T-RF patterns for the four microcosm treatments are shown in Fig. 2. Estimates of mean AM diversity at the microcosm level (H_{mic}) were similar at 3.99 and 3.95 using the restriction enzymes *Hin*fI or *Hsp*92II (Table 2), and the estimates of total diversity across all microcosms (H_{tot}) ranged from 15.7 to 16.1 (Table 2). Overall, only one-quarter of the total diversity was found within individual microcosm treatments; three-quarters was found among microcosms (Table 2), suggesting that the different plant assemblages in the microcosms are a major influence on the AM fungal community. On average, the minimum number of AM fungi colonizing the *P. lanceolata* roots was $145/(12 \times 2 \times 2) = 3.02$, where the numerator is the total number of detected T-RFs and the denominator is the 12 plants studied plus two enzymes plus two ends. We estimate that the minimum number of AM fungi in *P. lanceolata* bioassay roots is 4.3, 3.7, 3.1 and 1.0 for plants grown in bare soil, *C. flacca*, 12 plant species and *F. ovina* microcosms, respectively.

Table 1 Biomass of shoots of established plant assemblages, soil pH, total soil carbon, soil N and P and soil C : N ratio in the microcosms

Microcosm treatment	Shoot biomass (g d. wt m^{-2})	Soil properties pH (in H_2O)	Total C (mg g^{-1} d. wt)	Total N (mg g^{-1} d. wt)	C : N ratio	Total P (mg g^{-1} d. wt)
Bare soil	0 d (± 0.0)	6.2 b (± 0.04)	51 b (± 0.4)	5.3 c (± 0.05)	9.6 b (± 0.09)	0.73 a (± 0.02)
Carex flacca	20.1 c (± 2.3)	6.3 ab (± 0.10)	68 a (± 3)	6.0 bc (± 0.14)	11.3 a (± 0.28)	0.71 a (± 0.01)
Festuca ovina	36.0 ab (4.7)	6.3 ab (± 0.04)	67 a (± 3)	6.4 ab (± 0.10)	10.5 a (± 0.17)	0.76 a (± 0.04)
12 species	56.1 a (± 11.9)	6.5 a (± 0.07)	72 a (± 4)	6.8 a (± 0.29)	10.6 a (± 0.21)	0.71 a (± 0.01)

Data are mean (± SE), $n = 4$). Values sharing a letter are not significantly different (Tukey test, $P > 0.05$).

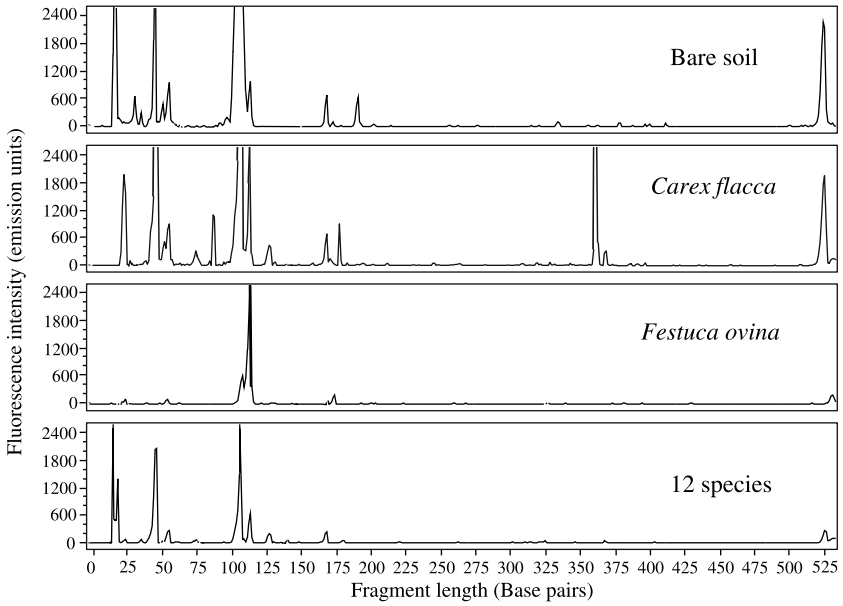

Fig. 2 Typical terminal-restriction fragment (T-RF) patterns of arbuscular mycorrhizal (AM) fungi in roots of *Plantago lanceolata* from grassland microcosms (digestions of the SSU rRNA gene fragments using *Hin*fI, forward strand). Fluorescence intensity (*y*-axis) is given in arbitrary units and T-RF sizes (*x*-axis) are given in base pair. Only peaks between 45 bp and 450 bp were used in the analysis.

Table 2 Estimates of the proportion of arbuscular mycorrhizal (AM) fungal diversity colonizing roots of *Plantago lanceolata* bioassay plants within and between microcosms

	H_{mic}	H_{tot}	H_{mic}/H_{tot}	$(H_{tot} - H_{mic})/H_{tot}$
*Hsp*92II	3.997	16.083	0.249	0.751
*Hin*fI	3.951	15.688	0.252	0.748
Mean	3.974	15.886	0.250	0.750

H_{mic}, average AM diversity in *P. lanceolata* roots from the different microcosms; H_{tot}, estimate of the overall AM diversity within *P. lanceolata* roots; H_{mic}/H_{tot}, proportion of AM diversity in *P. lanceolata* roots within microcosm treatments; $(H_{tot} - H_{mic})/H_{tot}$, proportion of diversity between microcosm treatments.

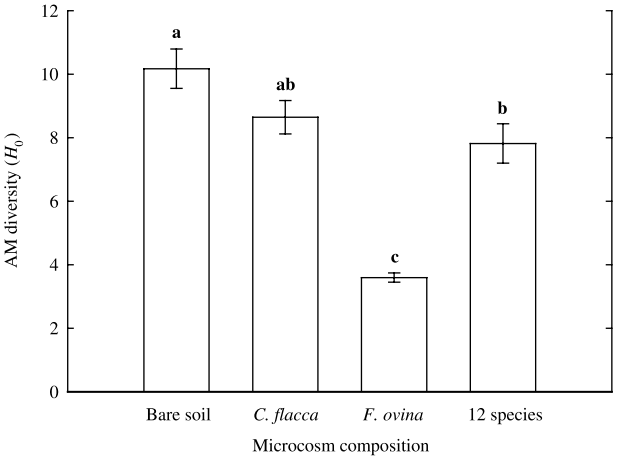

Fig. 3 Mean diversity of arbuscular mycorrhizal fungi (H_0) colonizing the roots of *Plantago lanceolata* bioassay seedlings removed from grassland microcosms after 12 wk of growth (± SE; $n = 3$). Bars with the same letter are not significantly different ($P > 0.05$).

The mean diversity of AM fungi (H_0) showed large and significant differences ($P < 0.001$) between plant assemblages (Fig. 3). Contrary to our initial hypothesis, the AM diversity was highest in the bare soil treatment ($H_0 > 10$), and was lowest ($H_0 < 4$) in the *F. ovina* monoculture (Figs 2 and 3). The AM diversity in *P. lanceolata* from the 12 species microcosms was significantly greater than in the *F. ovina* treatment but significantly less than in the bare soil. In the *C. flacca* treatment, the AM diversity in the bioassay seedlings was not significantly different from that in the bare soil or the 12-species mixture (Fig. 3).

The plant assemblages had a major effect on the AM community composition because the indices of similarity (S) of AM fungal diversity signatures (i.e. pairwise comparisons among T-RF patterns) were much higher within microcosm treatments ($S = 0.59–0.79$) than between them ($S = 0.31–0.41$; Table 3). As expected, the greatest value of similarity for AM in bioassay seedlings within microcosm treatments was highest ($S = 0.79$) for the assemblages with very low diversity, which were found in the seedlings grown with *F. ovina*. The smallest value ($S = 0.59$) was in the seedlings grown in the bare soil, which contained the greatest AM diversity (Table 3, Fig. 3).

The parsimony analysis provided further confirmation of effects of plant assemblages on the AM communities as each of the microcosm treatments was found to have developed consistent and distinct AM fungal communities in the roots of the *P. lanceolata* seedlings (Fig. 4).

Effect of plant assemblages on growth and nutrition of *P. lanceolata* bioassay seedlings

The mean dry weights of *P. lanceolata* shoots ranged from 22.4 mg in the 12-species mixture to 113.4 mg in the *C. flacca*

Table 3 Indices of similarity (*S*) of arbuscular mycorrhizal (AM) fungal diversity signatures (terminal-restriction (T-RF) patterns) among *Plantago lanceolata* bioassay root fragments within microcosm treatments (bold) (n = 3) and between microcosm treatments (± SE) (n = 6)

Microcosm	Bare Soil	*Carex flacca*	*Festuca ovina*	12 species
Bare soil	**0.59 ± 0.08**	0.36 ± 0.04	0.40 ± 0.02	0.31 ± 0.05
C. flacca		**0.67 ± 0.02**	0.35 ± 0.02	0.41 ± 0.05
F. ovina			**0.79 ± 0.01**	0.38 ± 0.02
Twelve species				**0.63 ± 0.02**

Table 4 Shoot dry weight (antilogarithms of means), N and P concentration and N : P ratio in *Plantago lanceolata* seedlings removed after 12 wk of growth in grassland microcosms

Microcosm treatment	Shoot d. wt (mg)	Shoot N (mg g^{-1} d. wt)	Shoot P (mg g^{-1} d. wt)	Shoot N : P ratio
Bare soil	55.1 a (± 13.1)	49.0 a (± 2.0)	1.51 a (± 0.12)	34.0 a (± 2.7)
Carex flacca	113.4 a (± 12.8)	48.5 a (± 2.6)	1.55 a (± 0.13)	34.1 a (± 3.4)
Festuca ovina	61.7 a (± 11.4)	25.2 b (± 1.9)	0.98 a (± 0.07)	26.0 ab (± 0.9)
12 species	22.4 b (± 11.1)	19.6 b (± 1.2)	1.13 a (± 0.10)	18.1 b (± 1.3)

Data are means (± SE), n = 4). Values sharing a letter are not significantly different (P > 0.05).

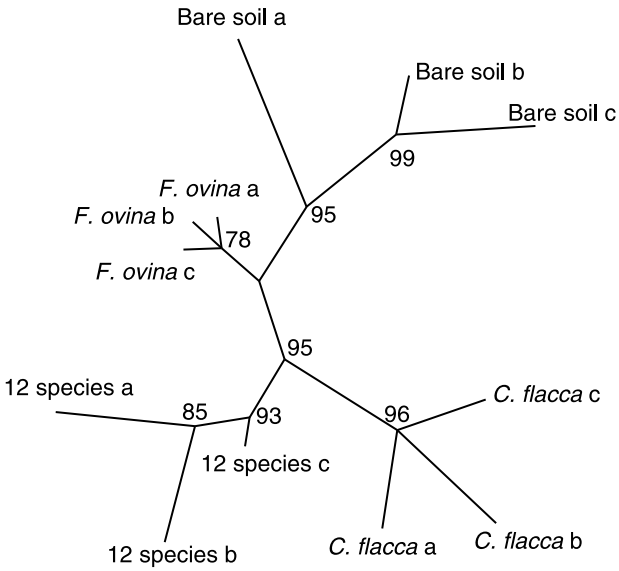

Fig. 4 Most parsimonious unrooted tree showing differentiation among arbuscular mycorrhizal fungal community in roots of *Plantago lanceolata* bioassay seedlings according to plant microcosm composition (a–c represent replicate microcosms). The tree was computed in PAUP 3.1.1 using the 'Branch and Bound' algorithm, and bootstrap values at the nodes were estimated from 500 replicates.

monocultures (Table 4). The biomass of the shoots from the 12-species mixture was significantly smaller than from plants grown in all other microcosm treatments. The shoot N concentrations ranged from 19.6 to 49 mg g d. wt^{-1} and shoot P concentrations from 0.98 to 1.55 mg g d. wt^{-1}. The shoot N and P concentrations in seedlings from the bare soil treatment were similar to those from the *C. flacca* monoculture, while those from the *F. ovina* monoculture fell between those from the *C. flacca* and 12-species mixture.

Both shoot N and P concentrations increased linearly with seedling biomass (Fig. 5a,b), the strongest relationship being for N concentration (y = 8.31 + 16.4x; R^2 = 55.4, P = 0.001; Fig. 5a). The shoot N : P ratios of the seedlings ranged from 18.1 to 34.1, with the smallest values recorded in the 12-species mixture and the highest in the bare soil and *C. flacca* treatments (Table 4).

Factors affecting AM diversity and shoot N and P concentration and biomass of *P. lanceolata* seedlings

The composition of the plant assemblages in the microcosms affected both their above-ground biomass (Table 1) and the growth of the bioassay seedlings (Tables 4 and 5). The ANCOVA showed that *P. lanceolata* biomass was significantly affected only by treatment group (P = 0.0009), and not by total species biomass or AM diversity (Table 5). The *P. lanceolata* shoot N concentrations were significantly positively affected by both AM diversity (P = 0.0015; Table 5; see also Fig. 6a) and *P. lanceolata* biomass (P < 0.0001; Table 5 and Fig. 5a). The *P. lanceolata* shoot P concentrations were also significantly positively affected by both AM diversity (P = 0.0059; Table 5 and Fig. 6b) and *P. lanceolata* biomass (P = 0.0185; Table 5 and Fig. 5b). This analysis found highly significant effects (P < 0.001) of the plant assemblages on AM diversity but did not detect any significant effect of the biomass of

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Table 5 Summary table of the ANCOVA outputs from general linear models used to test the significance of factors affecting the diversity of arbuscular mycorrhizal (AM) fungi, biomass and shoot N and P concentrations (dependent variables) of *Plantago lanceolata* bioassay seedlings grown in grassland microcosms

Dependent variables	Factors AM diversity (H_0) in *P. lanceolata* roots	Log_{10} *P. lanceolata* biomass	Log_{10} microcosm shoot biomass	Microcosm treatment
AM diversity (H_0) in *P. lanceolata* roots	–	ns	ns	$P < 0.0001$; $F_{2,5} = 97.39$
Log_{10} *P. lanceolata* biomass	ns	–	ns	$P = 0.0009$; $F_{3,12} = 10.96$
Shoot N concentration	$P = 0.0015$; $F_{1,8} = 22.23$	$P < 0.0001$; $F_{1,8} = 66.34$	ns	ns
Shoot P concentration	$P = 0.0059$; $F_{1,9} = 12.86$	$P = 0.0185$; $F_{1,9} = 8.23$	ns	ns

ns, Factors removed from model due to no significance.

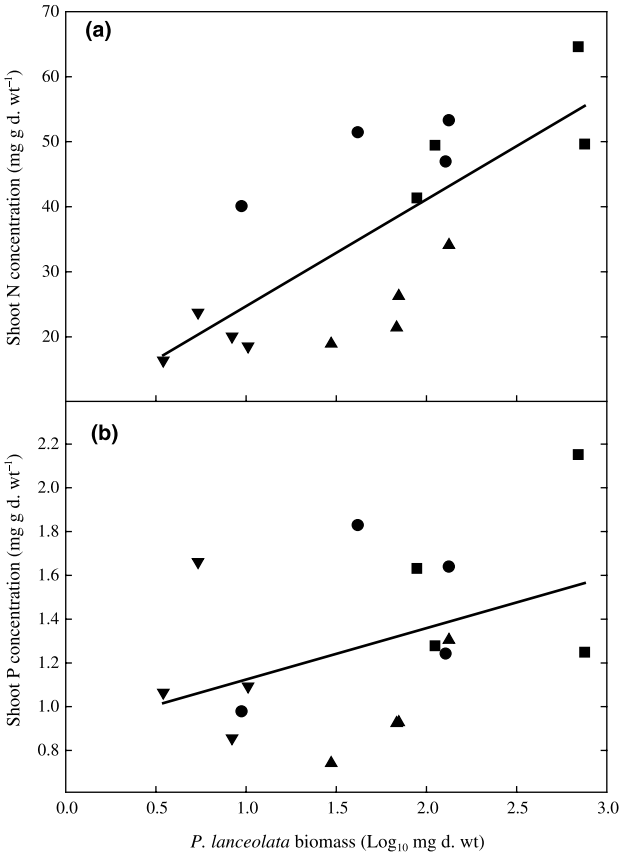


Fig. 5 Relationship between biomass of *Plantago lanceolata* bioassay seedlings and (a) shoot N concentration ($y = 8.31 + 16.4x$; $R^2 = 55.4\%$; $P = 0.001$) and (b) shoot P concentration ($y = 0.89 + 0.24x$; $R^2 = 17.3\%$; $P = 0.109$) grown for 12 wk in bare soil (circles), *Carex flacca* monocultures (squares), *Festuca ovina* monocultures (upward-pointing triangles) and a mix of 12 species (downward-pointing triangles). Regression lines do not account for potentially confounding effects of arbuscular mycorrhizal diversity in *P. lanceolata* roots, *P. lanceolata* biomass, microcosm shoot biomass and microcosm composition (see Table 5), but indicate the direction of the responses.

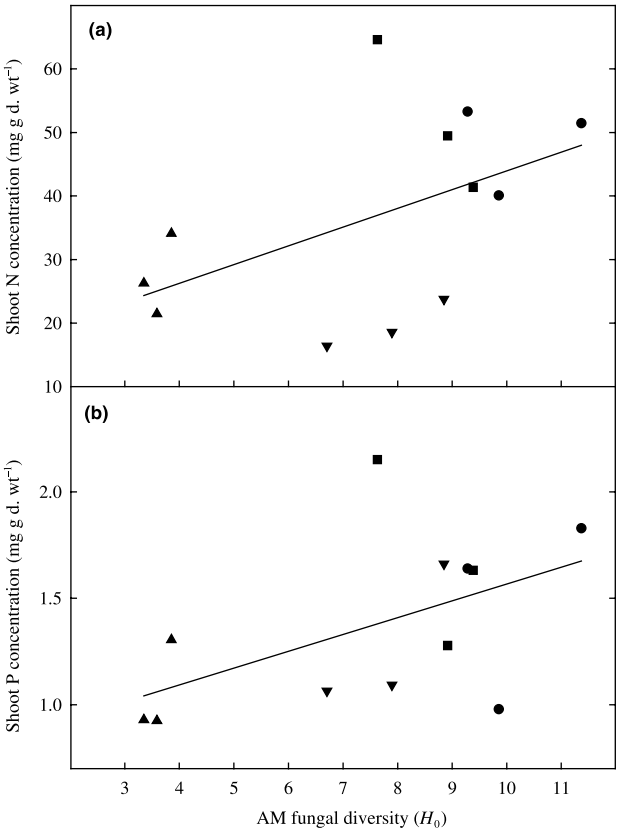


Fig. 6 Relationships between arbuscular mycorrhizal (AM) fungal diversity and (a) shoot N concentration ($y = 3.2x + 12.55$, $R^2 = 29.2\%$, $P = 0.070$) and (b) shoot P concentration ($y = 0.093x + 0.668$, $R^2 = 38.2\%$, $P = 0.031$) of *Plantago lanceolata* bioassay seedlings grown for 12 wk in grassland microcosms (circles, bare soil; squares, *Carex flacca* monocultures; upward-pointing triangles, *Festuca ovina* monocultures; downward-pointing triangles, a mix of 12 species). Regression lines do not account for potentially confounding effects of *P. lanceolata* biomass, microcosm shoot biomass and microcosm composition (see Table 5) but indicate the direction of the responses.

either the established plant assemblages or the *P. lanceolata* seedlings (Table 5).

There was a weak and nonsignificant ($P > 0.05$) correlation between shoot N concentration in the *P. lanceolata* seedlings and AM fungal diversity (Fig. 6a). However, there was a stronger ($R^2 = 38.7\%$) and significant ($P < 0.05$) positive correlation between shoot P concentration in the bioassay seedlings and AM fungal diversity (Fig. 6b).

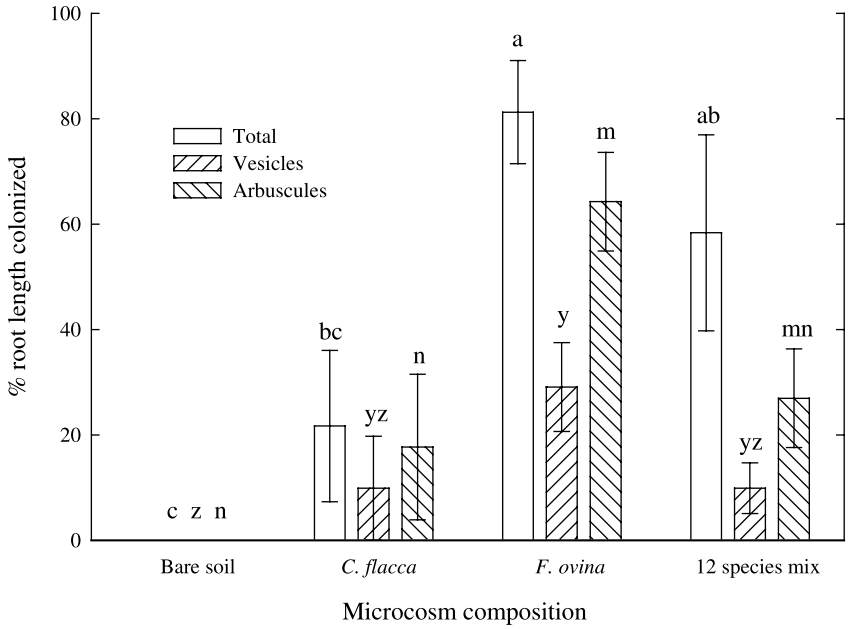


Fig. 7 Percentage of root length colonized by arbuscular mycorrhizal fungi and percentage occupancy by arbuscules and vesicles in *Plantago lanceolata* bioassay seedlings grown in microcosms containing bare soil, monocultures of *Carex flacca* and *Festuca ovina* and a mix of 12 species (Experiment 2; ± SE; $n = 4$). Bars with the same letter are not significantly different ($P > 0.05$). Total colonization, arbuscules and vesicles were analysed separately.

The effect of plant assemblages on AM colonization (Experiment 2)

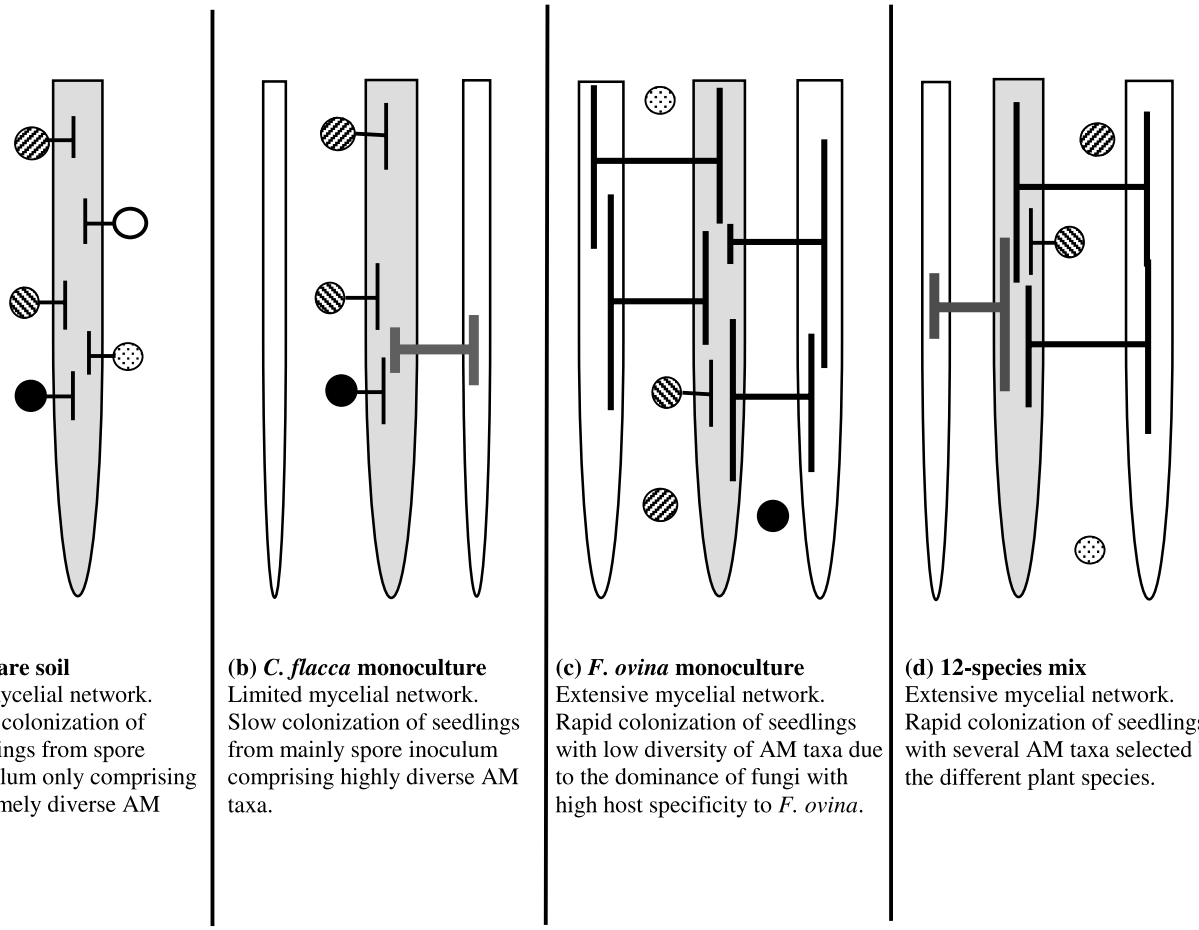
In the second experiment, 3-wk-old bioassay seedlings remained uncolonized in the bare soil treatments (Fig. 7). However, a similar preliminary bioassay experiment using replicate *P. lanceolata* seedlings grown in only one of the microcosms revealed that 28% and 44% of their roots became colonized by AM fungi after 6 wk and 9 wk growth, respectively.

In the 3-wk-old seedlings, the root length colonized by AM fungi was 20% in the *C. flacca* monocultures but this was not significantly different from that recorded in the bare soil treatment (Fig. 7). In the *F. ovina* monoculture, the root length colonized was 80% and this was significantly greater than both the bare soil and *C. flacca* treatments. Mean root length colonized in the 12-species mix, which had a high associated standard error, was 60% and was significantly greater than colonization measured from the bare soil treatment only. A similar pattern was seen for the abundance of vesicles and arbuscules (Fig. 7). No vesicles or arbuscules were seen in plants removed from the bare soil microcosm. The root length occupied by vesicles was 10% in both the *C. flacca* and 12 species mixture microcosms and increased significantly to approximately 30% in the *F. ovina* microcosm. The root length occupied by arbuscules was 20% and 30% in the *C. flacca* and 12-species mixture microcosms and increased significantly to 60% in the *F. ovina* microcosm.

Discussion

The application of the T-RFLP method demonstrated that AM diversity in the bioassay seedlings was affected by the composition of plant assemblages, even though these treatments were assembled from co-occurring plants transplanted from the same species-rich limestone grassland into natural soil. Our results show marked effects of the plant assemblages on both the diversity and composition of AM fungal communities colonizing the bioassay seedlings. Our hypothesis that AM fungal diversity would be less in monocultures of the AM-compatible host *F. ovina* than the 12-species mixture of mainly AM-compatible plants was supported by the data. However, contrary to our hypothesis that the least AM diversity would be found in seedlings grown in bare soil and in microcosms containing an established turf of the nonAM compatible species *C. flacca*, these treatments gave the greatest diversity.

This unexpectedly high diversity is likely to reflect differences in the modes of colonization and sources of inoculum in the bare soil and sedge treatments compared with the assemblages containing established AM-compatible plants. We propose (Fig. 8) that the observed differences between the AM diversities arise as a consequence of the predictably distinctive forms and vigour of the inoculum in the various treatments. The reduced rates of colonization of bioassay seedlings observed in the bare soil and *C. flacca* treatments, compared with those in *Festuca* and mixed species assemblages, is likely to arise from the fact that, in the former case (Fig. 8a,b), inoculum would be in the form of isolated propagules. In the latter case (Fig. 8c,d), colonization would arise through contacts between the growing roots of the *Plantago* seedlings and an established AM mycelial network in which triggering of spore germination and selection for compatibility and vigour had already taken place. Because propagules in the bare soil or *Carex* microcosms would not have been exposed to these



(a) Bare soil
No mycelial network. Slow colonization of seedlings from spore inoculum only comprising extremely diverse AM taxa.

(b) *C. flacca* monoculture
Limited mycelial network. Slow colonization of seedlings from mainly spore inoculum comprising highly diverse AM taxa.

(c) *F. ovina* monoculture
Extensive mycelial network. Rapid colonization of seedlings with low diversity of AM taxa due to the dominance of fungi with high host specificity to *F. ovina*.

(d) 12-species mix
Extensive mycelial network. Rapid colonization of seedlings with several AM taxa selected by the different plant species.

Fig. 8 Representation of the modes of colonization and the genetic diversity of AM fungi in *P. lanceolata* bioassay seedling roots (shaded) in the four microcosm treatments (a–d). Circles represent spore inocula, shading of hyphae and fill patterns of spores represent AM fungal types and root width represents plant species.

triggering or selection processes, the loss of diversity would be avoided. In this case, assuming as seems likely, that their longevity enabled them to survive from the time of the original soil sampling, a greater range of taxa would be available to colonize, albeit more slowly, the bioassay plants. Had the AM colonization of *P. lanceolata* bioassay plants been passive and stochastic, the AM fungal communities would have similar indices of similarity (*S*) both within and between microcosms.

In addition to the effects on diversity, we found strong AM fungal community differentiation in response to the composition of plant assemblages, which is consistent with the emerging evidence of some host-specificity in AM fungi in species-rich grasslands (Vandenkoornhuyse *et al.*, 2002b, 2003). As expected, the roots of *C. flacca* had little AM colonization but instead were mainly colonized by dark septate (DS) fungi (data not shown), as seen in other species of *Carex* (Read & Haselwandter, 1981). These DS fungi are likely to have established a network throughout the *C. flacca* monocultures. While there was no significant difference in AM diversity in seedlings grown in the bare soil and *C. flacca* monoculture, the AM communities developed in these treatments were distinct and placed in different clusters of the parsimony tree (Fig. 4). This raises the interesting prospect that both host and nonhost plants can exert an influence on the composition of AM communities, perhaps through effects on longevity and infectivity of propagules or through interactions between AM and DS fungi.

The longevity of the inoculum in the bare soil treatment is itself noteworthy because the soil was maintained free of plants throughout the 3-yr establishment phase of the microcosms. Clearly, some propagules, probably spores, were able to survive this period of incubation. Previous studies have shown that AM mycelium in dried root fragments stored for 6 months can retain viability and infect roots on rewetting (Tommerup & Abbott, 1981), but little is known about the longevity of spores. In addition, the process of coarse sieving of the soil that was placed in the microcosms is likely to have had a significant negative impact on the AM mycelial networks, as judged from previous studies of such disturbance (Merryweather & Fitter, 1998).

The *F. ovina* monoculture and 12-species mixture treatments contained plants with root systems heavily colonized

by AM fungi. Because it has been demonstrated that mycorrhizal host-plant preferences exist (Helgason *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002b, 2003), we would expect less AM diversity in *P. lanceolata* seedlings grown in the *F. ovina* monoculture microcosms compared with the microcosms with 12 plant species. Our T-RFLP results confirm this and provide strong evidence that differences in AM fungal community composition in the microcosms are strongly influenced by the host plants. In natural grassland ecosystems, where almost all plants are heavily colonized, we would expect AM fungal diversity to decrease if plant diversity decreases. This also explains why in roots of crop plants (i.e. monocultures) little AM fungal diversity has been observed (Helgason *et al.*, 1998; Daniell *et al.*, 2001) compared with the roots of plants collected from species-rich semi-natural ecosystems (Helgason *et al.*, 1999; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002b, 2003).

While it is not possible to enumerate precisely the AM species in the seedling roots from the T-RFLP data, the estimates of approximately 16 AM fungal types across the treatments is not dissimilar from recent records of AM species diversity in permanent grassland. For example, 24 phylogenetic species of AM fungi were recorded in an upland grassland by Vandenkoornhuyse *et al.* (2002b), and Klironomos and Hart (2002) recorded 10 species of AM from a single, old field in Canada. It is unlikely that any of the fragments encoded in the datasets resulted from partial digestions because replicates of the T-RFLP analyses were very similar within microcosm treatments (except for peak height). In addition, the specificity of the primer set we used in the PCR amplifications has been repeatedly shown to be very good (Helgason *et al.*, 1999; Daniell *et al.*, 2001; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002b, 2003). Furthermore, negative controls, using DNA from plants uncolonized by AM fungi, did not generate any amplification product, providing strong evidence that all T-RFs are from AM fungal genetic material.

Plantago lanceolata growth was strongly influenced by the species composition of the microcosms (Tables 4 and 5). The differences between treatments in *P. lanceolata* shoot biomass were extremely large; the biomass of the seedlings from the *C. flacca* monocultures being about five times greater than that from the mixed species microcosms after 12 wk of growth. Unexpectedly, the shoot N and P concentrations in the *P. lanceolata* seedlings increased with plant biomass (Table 5, Fig. 5). We would normally expect the greater biomass to result in dilution in the concentrations of these nutrients. We also found that the diversity of AM fungi associated with their roots showed significant positive correlations with shoot N and P concentrations (Fig. 6), with the plants supporting more diverse AM fungal communities tending to have greater concentrations.

These results provide some support for the observations of van der Heijden *et al.* (1998b) who demonstrated a positive correlation between increasing diversity of (artificially manipulated) AM fungal communities and shoot P concentrations in old field systems. In the present study, the growth and nutrition of the *Plantago* seedlings may have been affected by factors that were not included in the ANCOVA. For example, seedling growth is clearly influenced by the amount of photosynthetically active radiation reaching the plant shoots, which could have differed between the microcosm treatments. While this cannot be entirely discounted as an explanation for the growth responses of the *Plantago* seedlings, it is unlikely to have been a major factor because we found no influence of microcosm shoot biomass on the above-ground biomass of the bioassay plants harvested from them (Table 5, $P = 0.3187$).

Clearly, however, although the biomass of the surrounding vegetation may be a good surrogate for some potentially influential confounding factors, such as the amount of photosynthetically active radiation reaching the *P. lanceolata* seedlings, it may not be entirely satisfactory for others, such as differences in nutrient scavenging activities of roots. It is possible that the associated decrease in shoot N and P concentrations in the *P. lanceolata* seedlings reflects a greater intensity of competition for nutrients from the established plants. This is supported by the observation that the greatest P concentrations were found in the seedlings grown in bare soil or with the non-mycotrophic *C. flacca.* While *C. flacca* lacks extensive mycorrhizal colonization, it produces specialized dauciform cluster roots that are implicated in mobilization of sparingly soluble sources of P (Ballard, 2001). This distinct mode of acquisition may mean that competition for P with *Plantago* will be less intense than in cases where the bioassay seedlings are sharing linked mycorrhizal associations with *F. ovina* or the 12-species mixtures of plants. Here, both the seedlings and established plants are likely to be exploiting the same P sources via a common mycelial network.

Furthermore, T-RFLP analysis does not enable us to identify the species of fungi that were colonizing the *P. lanceolata* bioassay seedlings because SSU rRNA gene sequences from named cultures available in international databases do not accurately reflect the distribution patterns of AM fungi in nature. Therefore, we do not know for certain if the greater shoot N and P concentrations in some of the *P. lanceolata* seedlings were a consequence of either greater AM diversity *per se* or an increased likelihood of the presence of AM fungi more effective at acquiring P. In the future, this question could be addressed by sequencing the amplified SSU rRNA genes of the AM fungal communities colonizing the roots of bioassay seedlings.

The influence of the treatments on other components of the soil microbial community might also have affected the diversity and functioning of AM fungi. For example, using the same microcosms, Johnson *et al.* (2003) demonstrated that plant community composition had a marked effect on the activity and diversity of heterotrophic microorganisms. Further work is clearly required to understand more fully the mechanisms by which the identity and diversity of plant

functional type affect key aspects of ecosystem functioning including the activity and diversity of free-living and symbiotic microorganisms.

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