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Impact of soil warming and shading on colonization and community structure of arbuscular mycorrhizal fungi in roots of a native grassland community

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Abstract

Arbuscular mycorrhizal (AM) fungi have a major influence on the structure, responses and below-ground C allocation of plant communities. Our lack of understanding of the response of AM fungi to factors such as light and temperature is an obstacle to accurate prediction of the impact of global climate change on ecosystem functioning. In order to investigate this response, we divided a grassland site into 24 plots, each either unshaded or partly shaded with soil either unheated or heated by 3 °C at 2 cm depth. In both short-term studies in spring and autumn, and in a 1-year-long study, we measured root length colonization (L_RC) by AM and non-AM fungi. For selected root samples, DNA sequences were amplified by PCR with fungal-specific primers for part of the small sub-unit (SSU) rRNA gene. In spring, the total L_RC increased over 6 weeks from 12% to 25%. Shading significantly reduced AM but increased non-AM fungal colonization, while soil warming had no effect. In the year-long study, colonization by AM fungi peaked in summer, whereas non-AM colonization peaked in autumn, when there was an additive effect of shading and soil warming that reduced AM but increased non-AM fungi. Stepwise regression revealed that light received within the 7 days prior to sampling was the most significant factor in determining AM L_RC and that mean temperature was the most important influence on non-AM L_RC. Loglinear analysis confirmed that there were no seasonal or treatment effects on the host plant community. Ten AM fungal sequence types were identified that clustered into two families of the Glomales, Glomaceae and Gigasporaceae. Three other sequence types were of non-AM fungi, all Ascomycotina. AM sequence types showed seasonal variation and shading impacts: loglinear regression analysis revealed changes in the AM fungal community with time, and a reduction of one *Glomus* sp. under shade, which corresponded to a decrease in the abundance of *Trifolium repens*. We suggest that further research investigating any impacts of climate change on ecosystem functioning must not only incorporate their natural AM fungal communities but should also focus on niche separation and community dynamics of AM fungi.

Keywords: arbuscular mycorrhizas, grassland community, RFLP, shading, soil warming, SSU rRNA

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Introduction

Grasslands cover nearly 20% of the land surface (Parton *et al*., 1995), occur in nearly all climatic zones, and contain >10% of global soil carbon stocks (Eswaran *et al*., 1993). Most primary production in grasslands occurs below ground in root systems and their associated symbionts (Jackson *et al*., 1996), and temperate grasslands can maintain a high organic soil C-content of around 150 t ha^{-1} with only 0.5 t ha^{-1} biomass (Killham, 1994). Virtually all grassland plant species

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form arbuscular mycorrhizas (AM; Miller, 1987), and they can act as a sink for up to 15% of primary production (Miller & Kling, 2000). In understanding the response of grasslands to changing climates the response of AM fungi to key environmental factors, such as temperature and radiation, needs to be quantified.

Global mean temperature is expected to increase by 1.4–5.8 °C over the next century (Houghton *et al*., 1995, 2001), possibly leading to changes in cloudiness (Wielicki *et al*., 1995); a warmer atmosphere will contain more water vapour, which is likely to increase cloudiness in at least some parts of the world (Houghton *et al*., 2001), which in turn will affect photosynthesis. Few studies on the AM symbiosis have considered these climate predictions, despite its ecological importance. AM fungi are obligate symbionts (Cooper, 1984), receiving their carbohydrates from the host plant in exchange for enhanced nutrient uptake (mostly phosphate) or other benefits (Koide, 1991; Newsham *et al*., 1995). Any impact on host plant photosynthesis may therefore indirectly affect AM fungal growth and thus carbon allocation into the rhizosphere via the extraradical mycelium (ERM) (Jakobsen & Rosendahl, 1990), with consequential impacts on other soil biota (Bonkovski *et al*., 2000; Mar Vazquez *et al*., 2000). AM colonization can also enhance host photosynthesis and sometimes plant biomass production (Smith & Read, 1997) mainly due to a positive feedback following increased carbon sink strength of roots (Harris & Paul, 1987; Wright *et al*., 1998a), especially under elevated CO_2 concentrations (Fitter *et al*., 2000). This feedback will increase carbon allocation to both litter and root systems. Consequently, this symbiosis is uniquely important in linking above- and below-ground carbon cycling (Norby & Jackson, 2000). However, most experiments examining AM colonization effects on below-ground carbon allocation suffer from obtaining suitable controls as observed by Wright *et al*. (1998a), and our knowledge about responses under natural conditions is rather scarce (Smith & Read, 1997).

Increased root length colonization (L_RC) has been observed under both elevated CO_2 and soil temperature (Fitter *et al*., 2000), but can normally be explained as a consequence of greater plant size (Staddon & Fitter, 1998). However, light quantity is likely to be of greater influence than small changes in soil temperature as it directly determines L_RC (Daniels Hetrick, 1984) via photosynthesis, and thus root growth (Fitter *et al*., 1998) and carbohydrate transfer to the fungus (Cooper, 1984; Tester *et al*., 1985). Seasonal light change determines the plant's growing season and might thus be more influential than shading on AM growth in the field as shoots acclimate to constant shading (Murchie & Horton, 1997) with increased specific leaf area (Metcalfe *et al*., 1998). There is a clear need to simulate changes of global climate in field studies and to determine AM fungal responses both individually and as a community (Miller & Kling, 2000), as these fungi have a powerful impact on plant growth (Abbott & Robson, 1984) and fungal-specific responses might alter plant communities (van der Heijden *et al*., 1998). However, despite their important role in almost all ecosystems, the ecology of AM fungi, and in particular their responses to key environmental factors (e.g. temperature and light), remain obscure, although necessary in order to predict impacts of a changing climate at an ecosystem level.

We have measured the effects of shade and soil warming on L_RC of AM and non-AM fungi in an artificial native grassland community. As the chosen site was formerly used as an arable plot, we expected low AM fungal diversity (Giovannetti & Gianinazzi-Pearson, 1994; Helgason *et al*., 1998). This would make it easier to detect any seasonal or treatment effects and also impacts on key species. So far only around 150 species, all belonging to the order Glomales (Glomeromycota), have been described by spore morphology. Attempts to distinguish AM species based entirely on intra-radical hyphal structures (Abbott & Robson 1984; Merryweather & Fitter, 1998a) are system-specific and time consuming. More recently, fungal taxa have been recognized from differences in their small sub-unit (SSU) rRNA (Giovannetti & Gianinazzi-Pearson, 1994; Helgason *et al*., 1999), amplified directly from spores or from host plant roots using AM fungal-specific primers. A combination of molecular analysis with assessment of colonization of paired root samples over time enabled us to characterize both the structure and dynamics of the AM fungal community and also its interaction with other root-colonizing fungi. We tested four hypotheses: (i) light has a greater influence on root colonization by AM fungi than temperature within the range likely to be experienced following climate warming; (ii) seasonal light changes will be more important than shading; (iii) soil warming is most influential during cooler periods either by increasing colonization or by prolonging the production of young roots; (iv) there will be differences in the responses of native AM fungal species to temperature and shading treatments.

Materials and methods

Growth conditions

The experiment was carried out in the University of York experimental garden on a previously cultivated sandy loam (pH 6.5) soil. The site had received no fertilizers for many years, and was considered to

provide a medium nutrient status soil with an established AM fungal community. The grassland community was sown in August 1998 with a seed mix containing *Plantago lanceolata* (5% by weight), *Holcus lanatus* (5%), *Festuca rubra* (25%), *Trifolium repens* (8%), *Cynosurus cristatus* (45%) and *Agrostis capillaris* (12%) (all Emorsgate Seeds, Norfolk, UK); a number of volunteer species were also present but made no major contribution to the biomass (Edwards *et al*., in press). The site was initially dominated by *P. lanceolata*, but *H. lanatus* became the dominant species once occasional cutting, three times during the season, was implemented.

Experimental design

The field site (8.5 m × 14.0 m) was divided into 24 plots (split plots of 0.5 m × 1.0 m due to shading frames, see below), either unshaded (NS), lightly shaded (S/2) or deeply shaded (S), and soil either at ambient temperature (unheated, A) or heated (H). However, light conditions were measured at 75 cm and actual shading at leaf level was somewhat less. Each treatment combination had four replicates. For shading, a frame (1.0 m (height) × 1.5 m × 2.0 m) was covered by layers of green and black shade mesh (Tildenet, East Riding Horticulture, Sutton-on-Derwent, UK). Each frame shaded two plots without changing the light quality (measured with a PR1010 spectral radiometer, Macam Photometeric Ltd., Livingston, UK; E. Edwards, unpublished results). There were two photosynthetically active radiation (PAR) sensors per shading treatment. Soil temperature was regulated using a heating system consisting of a series of loops of a heating cable, separated by 5 cm, attached to metal grids (1.0 m × 0.5 m) on the soil surface, providing an array of 2.5 cm × 2.5 cm sampling quadrats. Soil warming controllers (one for each warming treatment) were designed and built by D. Benham and each used an array of thermistor probes (Delta-T Devices Ltd, Cambridge, UK) to maintain the temperature differential or maintain ambient temperature below shading. Heated plots were kept at 3 °C above ambient temperature, measured continuously in the ambient, unshaded plots at 2 cm soil depth; shaded plots were kept exactly at ambient temperature. A grid was laid in all 24 sub-plots irrespective of whether the soil was to be warmed. For further details of a similar heating system, see Ineson *et al*. (1998). There were three experimental periods: the first two sampling periods were short-term experiments (a: 15 March 1999–26 April 1999 with 48% (S/2) and 67% (S) shade; b: 15 September 1999–3 November 1999 with 46% (S/2) and 53% (S) shade) and the third was a year-long experiment (c: 26 January 2000–10 January 2001 with 70% (S/2) and 86% (S) shade); treatments started on (a) 19 March, (b) 23 September and (c) 28 January. Shading and soil warming treatments were not applied at other times. Shading treatments were chosen and adjusted to optimize plant growth effects (Edwards *et al*., in press). The values for the two shading treatments were predetermined by the experimental design. Soil cores (10 cm in depth, 2 cm diameter) were taken randomly from different 2.5 cm^2 sampling quadrats, refilling the volume with soil from the surrounding area and recording the number of stems of the three most abundant plant species (*P. lanceolata*, *H. lanatus* or *T. repens*) for each soil core. To avoid edge effects, only the inner 476 sampling quadrats were used for sampling, avoiding the outer 10 cm of each grid.

Collection of environmental data

For each plot, soil temperature and light levels were recorded every 30 min (averaging 1 min readings) throughout the experiment using a weather station (Delta-T Devices Ltd). In all three periods, soil heating continuously maintained temperature at a depth of 2 cm in soil 2.7 °C above ambient. The monthly mean temperatures and light conditions for the period 1961–1990 for northern England were obtained from the Meteorological Office website (www.metoffice.com). Shortly before and during the first spring period (a) (01 January–30 April 1999), surface temperatures were ca. +1.6 °C warmer than average and sunshine hours were ca. 115% of average. The autumn period (b) showed no major temperature or precipitation deviations. The winter months (1 November 1999–29 February 2000) before and at the beginning of period (c) were also warmer by ca. 1.5 °C at the soil surface and had higher sunshine hours of ca. 50% above average (1 December 2000–29 February 2001). Moreover, summer 2000 was exceptionally warm (ca. + 1.0 °C above the long-term mean at the soil surface over the period 1 May–30 August).

Fungal measurements

The length of roots colonized (L_RC) by AM and other (non-AM) fungi (n.b. saprotrophic and parasitic fungi were not distinguished) was estimated on roots extracted from the soil samples. Sampling during periods (a) and (b) was carried out weekly and during period (c) at about 5-week intervals, except during extreme weather conditions (i.e. snow cover or intense rainfall). Each soil core was placed on a mesh (0.75 mm pore size, diameter 20 cm, Endecotts Ltd, London, UK) and all roots were extracted. Root subsamples from a

single core were taken randomly, pooled together, stained and investigated for L_RC as described in Staddon *et al*. (1998), except that only roots of diameter <2 mm were taken. No distinction was made between roots of different species. Clearing in 10% KOH (ca. 10 min) and staining in 0.1% acid fuchsin (ca. 35 min) were conducted in a waterbath (75 °C). To improve staining results, samples were acidified with 1% HCl and stained twice. L_RC of AM and non-AM fungi were scored separately, as was L_RC of arbuscules (L_RC_{arb}) and vesicles (L_RC_{ves}). When both AM and non-AM colonization occasionally occurred in the same root intersection, they were recorded separately. L_RC was also expressed as a percentage of the total root length. Random samples of the remaining roots from each core of period (c) were washed in deionized water and dried on filter paper (Whatman® No. 1), placed into labelled Eppendorf tubes and stored in a freezer (-20 °C) until further molecular analysis. For this, only five sampling dates from period (c) were used: the first sample was collected on 26 January, the second and third samples were combinations of 10 May/7 June and the 27 September/25 October 2000, respectively. Choosing and pooling of samples was influenced mainly by observed L_RC patterns and also by the amount of roots per core.

L_RC data and statistical analysis

Statistical analysis was performed using SPSS v10.0 (SPSS Science, Birmingham, UK). All data were verified and transformed appropriately (e.g. arcsine transformation for percentage values and mostly log or square root transformation for other parameters; however, data were back-transformed in tables and figures) to normalize skewed distributions before statistical analysis and data were also verified for equality of variance (Levene's test in SPSS); no cases were encountered where data were unsuitable for ANOVA. Data were then tested for any treatment effects and interactions using the univariate command of the general linear model (three-way ANOVA) with sampling day, soil warming and shading treatment as independent factors. Soil temperature and PAR were calculated as means and sums for 1, 3, 7 and 14 day periods prior to the sampling time, respectively; period (c) was divided into a spring (c1: samples 1–4), summer (c2: samples 5–7) and autumn (c3: samples 8–11) period. Data sets were tested for correlations with any of the investigated L_RC parameters by performing a stepwise regression. Data of the period (c) were tested for any seasonal or treatment effect on the mean number of recorded plant species of corresponding soil cores (species as above) using a multinomial model of the general loglinear model with sampling time (1–3), soil warming (1, 2) and shading (1–3) as factors. For details of the analysis, see Helgason *et al*. (1999).

Molecular techniques

DNA was extracted from plant roots using a CTAB extraction method following Gardes & Bruns (1993), except freeze–thaw steps were omitted. Partial SSU DNA fragments (ca. 550 bp) were amplified using *Pfu* DNA polymerase (Promega, Madison, WI, USA) using a universal eukaryotic primer NS31 (Simon *et al*., 1992) and a general fungal primer AM1 (Helgason *et al*., 1998) designed to exclude plant DNA sequences. The reaction mix (50 μL) consisted of 0.2 mM dNTPs, 10 pmols of each primer and the corresponding reaction buffer. PCR was carried out for 29 cycles (one cycle at 94 °C for 3 min, 58 °C for 1 min and 72 °C for 1.5 min, and nine cycles at 94 °C for 45 s, 58 °C for 1 min and 72 °C for 1.5 min, and 19 cycles at 94 °C for 45 s, 59 °C for 1 min and 72 °C for 1.5 min) on a programmable heat block (PTC-100, MJResearch Inc., MA, USA). The resulting blunt-ended products were cloned into PCR-Script Amp SK(+) (Stratagene, La Jolla, CA, USA) and transformed into *Epicurian coli*^{R} (XL10-Gold^{TM}, Stratagene). It was intended to amplify twice from each replicate root sample and yield a minimum clone number of 20 per treatment and sampling time. However, two spring (ambient with 67% shade (AS) and heating with 48% shade (HS/2)) and one summer (ambient with 70% shade (AS/2)) amplifications failed completely, and due to low clone numbers spring samples ambient without shade (ANS) and heating without shade (HNS) were amplified three times. Further, for the autumn treatment heating with 46% shade (HS/2) the number of clones was only 19.

Putative transformants were screened by PCR using T3 and T7 primers. PCR products from up to 16 positive clones (size of approximately 750 kb) from each transformation were digested with the restriction enzyme *Hin*fI, according to the manufacturer's instructions (Promega). At least one clone of the 15 most common restriction fragment length polymorphism (RFLP) types (including three of clearly non-AM fungi e.g. size >800 kb) was sequenced on an ABI377 automated sequencer and a further 490 could then be classified by RFLP typing (the remaining clones were most likely non-AM fungi with very low frequencies). For sequencing, T3/T7-amplified PCR products were cleaned using PCR purification spin columns (Life Technologies, Paisley, UK) and sequenced according to the manufacturer's instructions using the Dye terminator cycle sequencing kit with AmpliTaqFS DNA

polymerase (ABI Perkin–Elmer, Norwalk, CI, USA), with AM1 as the sequencing primer.

Molecular data and statistical analysis

Related sequences were identified by a Blast search (http://www.ncbi.nlm.nih.gov/BLAST/blast_references.html). Clustal X (Thompson *et al*., 1997) was used for multiple alignment for a neighbour-joining phylogeny (Saitou & Nei, 1987). Data were tested (statistical package as above) for sampling time and treatment effects using a multinomial model of the general loglinear model with sampling time (1–3), soil warming (1, 2) and shading (1–3) and fungal groups as factors. Fungal groups were combined either according to neighbour-joining tree similarities and clone numbers or according to a cluster analysis with the *K*-mean test from the classify command. Data were also tested with the multidimensional scaling (MDS) command in SPSS to detect any other grouping patterns. For this procedure, the two shading treatments were combined giving four treatments in total, which were also defined by the recorded above-ground plant species (see experimental design).

Results

Effects on L_RC observed in spring and autumn (periods a and b)

Soil warming had no effect on the length of colonized roots (L_RC) of AM or non-AM fungi in either period. The total (both AM and non-AM fungi) L_RC (data not shown) was low in the spring period (a), increased over time from 12% to 25% and was significantly reduced by shading over the last three weeks under 67% shade ($F_{2,126} = 3.96$, $P = 0.021$), entirely because of an effect on AM fungi (Fig. 1a); the root length colonized by arbuscules (L_RC_{arb}) was usually less than 20% of AM L_RC, but was similarly reduced by shading ($F_{2,126} = 3.33$, $P = 0.039$).

In the first autumn period (b), the values of L_RC were higher than in spring (period a). Percentage L_RC of AM fungi increased from ca. 35% to over 60% (Fig. 1b), but the values of non-AM L_RC were <25%. The 63% (S) shade treatment significantly increased AM L_RC late in the season to about 58% relative to the unshaded plots (Fig. 1b), as there was a significant interaction with sampling time. A similar shading effect could be observed on L_RC_{arb} ($F_{2,144} = 3.76$, $P = 0.026$) and L_RC_{ves} ($F_{2,144} = 5.94$, $P = 0.003$), with a mean colonization of 40% and 50% (L_RC_{arb}) and 5% and 10% (L_RC_{ves}), in unshaded (NS) and S treatments, respectively. The mean AM L_RC in the first sample for the S treatment in the autumn period was low (Fig. 1b).

Effects on L_RC observed in the long-term experiment (period c)

The year-long period (c) showed an obvious seasonal pattern with AM L_RC peaking in summer (Fig. 2a, b) and non-AM LRC in autumn (Fig. 2c). Consequently, they were inversely correlated (Fig. 2d): whereas AM L_RC initially peaked at about 50%, non-AM fungi decreased from ca. 30% to about 15% and thereafter as AM L_RC decreased, non-AM L_RC increased again. Percentage AM L_RC was significantly affected by both shade (Fig. 2a) and soil warming (Fig. 2b); both reduced L_RC from June onwards, whereas in unshaded plots L_RC did not decrease before September (Fig. 2a). This

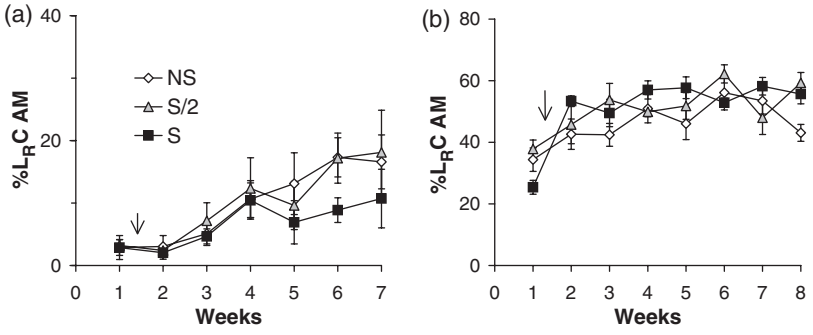

Fig. 1 Shading effects on mean percentage of root length colonized by arbuscular mycorrhizal (AM) fungi (%L_RC AM) during weekly samples in 1999 of (A) the spring period (a: 15 March–26 April) and (B) the first autumn period (b: 15 September–3 November) ± standard error (SE). Combined ambient and soil warming treatments are shown, as there were no significant soil warming impacts on AM L_RC during either period. Arrows indicate the beginning of treatments, which were: no shade (NS), 48% and 46% shade (S/2) and 67% and 63% shaded (S) for the spring and autumn period, respectively. Significances between shading treatments based on a three-way ANOVA were as follows: (A) $F_{2,126} = 4.13$, $P = 0.018$; (B) $F_{2,144} = 3.99$, $P = 0.021$; shading * harvest: $F_{14,144} = 1.90$, $P = 0.031$; there were no other significant differences for either treatment or interaction terms. Note the different *y*-axis scales between (A) and (B).

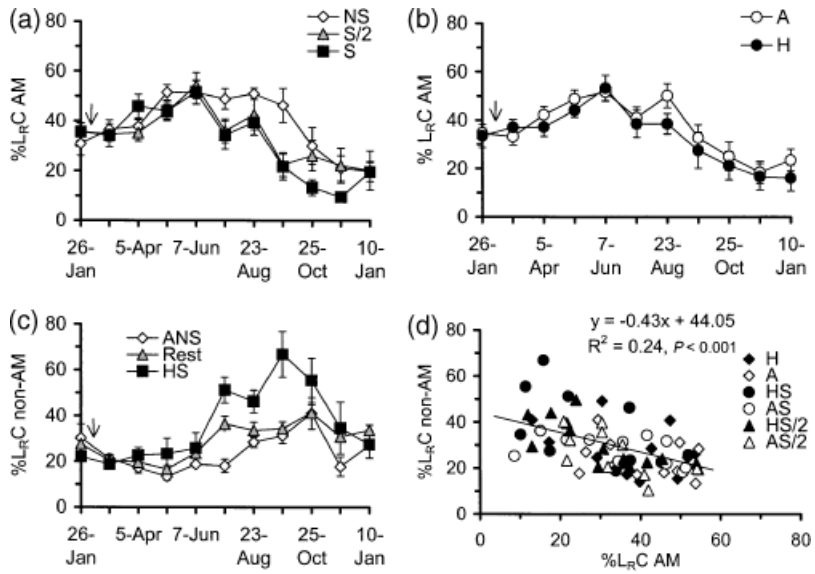

Fig. 2 (a) Effects of shading treatments ($F_{2,198} = 5.61$, $P = 0.004$) on mean percentage of colonized roots by arbuscular mycorrhizal (AM) fungi (%L_RC AM) during the year-long study ± standard error (SE); combined ambient (A) and soil warming (H) treatments are shown. (b) The impact of soil warming on AM L_RC was also significant ($F_{1,198} = 5.28$, $P = 0.023$). (c) Mean %L_RC for non-AM fungi (%L_RC non-AM) is shown as a combination of ambient and unshaded (ANS), soil warming with 86% shade (HS) and all other (rest) treatments; shading: $F_{2,198} = 6.63$, $P = 0.002$; soil warming: $F_{1,198} = 23.00$, $P < 0.001$; soil warming*sampling time: $F_{10,198} = 2.73$, $P = 0.004$. Arrows indicate the beginning of treatments, which were: no shade (NS), 70% shade (S/2) and 86% shaded (S). Significant differences between shading treatments were based on a three-way ANOVA; there were no other significant differences for either treatment or interaction terms. (d) Relationship between %L_RC of non-AM and AM fungi; the regression line was highly significant ($F_{1,262} = 88.83$, $P < 0.001$).

was also true for L_RC_{arb} (shade: $F_{2,198} = 9.54$, $P < 0.001$; heating: $F_{1,198} = 7.30$, $P = 0.007$). Non-AM L_RC was increased by both shading and soil warming (Fig. 2c); effects were additive as there was no interaction between them. However, there was an interaction between soil warming and sampling time as there were no effects at the beginning of the year (Fig. 2c). All these colonization patterns fell back to near initial levels in the final samples (Fig. 2).

Influence of soil temperature and light conditions prior to each sampling time and its effects on the host plant community

Multiple regression analysis (Table 1) showed that a PAR sum 1–3 days prior to sampling was the most important factor during the spring period (a); it increased AM but decreased non-AM L_RC, which was also true for the winter period of the year-long study (c1). In autumn periods, longer PAR sums of 7 days prior to sampling were influential: in the first autumn period (b), they decreased both non-AM L_RC and L_RC_{ves} and during the second period (c3), they increased all AM L_RC measures; during the full-year period (c), the PAR of the previous day was positively correlated with all AM measures. Soil temperature (summed over 14 days) affected AM L_RC only in the summer period (c2) and reduced both AM L_RC and L_RC_{arb}. Non-AM L_RC was reduced by temperature during the first autumn period (b), but increased with higher mean temperature during both summer and autumn periods (c1, c2) and during the entire period (c).

H. lanatus and *P. lanceolata* were the most abundant plant species in the ambient treatments. *T. repens* was less abundant and *P. lanceolata* more abundant in shaded treatments (Fig. 3), but these patterns were present before any treatments were applied (data not shown), for which no apparent reason could be found. The mean numbers for *H. lanatus* were 2.1 per core, with no treatment variations. A loglinear analysis was used to test for any treatment impacts on species counts; there were no significant seasonal or treatment impacts on the host plant community during period (c) as removing single factors or any of the interaction terms did not differ from the saturated loglinear model.

Indigenous AM fungal community and observed fungal groups

Alignments of the 19 partial SSU rRNA fragments with a selection of GenBank reference sequences produced a phylogenetic tree in which the majority of the

Table 1 Results of stepwise regression (SR) analysis of individual root colonization measures as a function of mean temperature and photosynthetically active radiation (PAR) sums received before sampling times

	Spring period (a)				Autumn period (b)				Winter period (c1)				Summer period (c2)				Autumn period (c3)				Entire Period (c)			
L_RC*	SR†	r^2‡	B§	P¶	SR	r^2	B	P	SR	r^2	B	P	SR	r^2	B	P	SR	r^2	B	P	SR	r^2	B	P
Total	–	–	–	–	PAR_7	0.110	−0.001	***	–	–	–	–	–	–	–	–	PAR_7	0.191	0.006	***				
	–	–	–	–	TEM_3	0.081	−0.020	**	–	–	–	–	TEM_1	0.106	0.025	**	TEM_{14}	0.221	0.020	***	TEM_{14}	0.290	0.020	***
Non-AM	PAR_3	0.041	−0.001	*	–	–	–	–	PAR_1	0.106	−0.007	**	–	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	TEM_3	0.037	−0.010	*	–	–	–	–	TEM_{14}	0.381	0.030	***	TEM_7	0.112	0.013	**	TEM_{14}	0.082	0.013	***
AM	PAR_1	0.085	0.004	***	PAR_7	0.080	−0.001	**	PAR_3	0.116	0.003	**	–	–	–	–	PAR_7	0.191	0.007	***	PAR_1	0.183	0.007	***
	–	–	–	–	–	–	–	–	–	–	–	–	TEM_{14}	0.104	−0.017	*	–	–	–	–	–	–	–	–
Arb.	PAR_1	0.079	0.004	**	–	–	–	–	–	–	–	–	–	–	–	–	PAR_7	0.197	0.007	***	PAR_{14}	0.181	0.007	***
	–	–	–	–	–	–	–	–	–	–	–	–	TEM_{14}	0.094	−0.017	*	–	–	–	–	–	–	–	–
Ves.	–	–	–	–	PAR_7	0.036	−0.001	*	–	–	–	–	–	–	–	–	PAR_1	0.113	0.019	**	PAR_1	0.051	0.019	***

Note that the entire period (c) was also analysed as individual periods (c1–c3).

*Root colonization (L_RC) by total fungi, non-AM fungi, AM fungi and arbuscular (Arb.) and vesicular (Ves.) colonization of AM fungi, respectively.

†PAR_n is the total PAR received, and TEM_n is the mean temperature over the n days immediately prior to sampling. In each case, $n = 1, 3, 7, 14$ were tested but only the most significant is shown.

‡Goodness of fit (r^2).

§Slope (B).

¶Significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

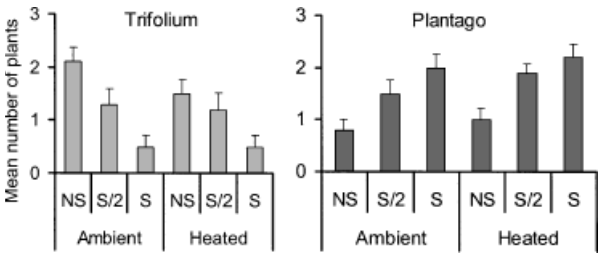

Fig. 3 Mean counts of the two plant species *Plantago lanceolata* and *Trifolium repens*, shown as the average of the sums of the four replicates per treatment over the 10 sampling times for the experimental period (c); (error bars indicate standard error, $N = 10$), where NS (nonshaded), S/2 (70% shade) and S (86% shade).

sequences clustered within the Glomales, and largely within well-supported groups corresponding to the two families, Glomaceae and Gigasporaceae (Fig. 4); no *Acaulospora* species were detected. Sequences were compared with those identified in previous studies from our laboratory (e.g. Helgason *et al*., 1998, 1999). There were ten AM sequence types (Fig. 4), yet only eight different RFLP types: sequence type Glo 45 and Glo 43 had the same RFLP type as sequences most similar to Glo 44 and Glo 4 sequences, respectively.

The three sequences that were detected throughout the year but showed no similarity to AM fungi were combined (Table 2), but excluded from the AM analysis. They were nearly identical to sequences of *Aniptodera chesapeakensis*, *Tetracladium marchalianum* and *Chaetomium globosum* (Fig. 4), all Ascomycetes. To this group were further added five non-sequenced RFLP patterns of obviously non-AM fungal SSU rRNA fragments (Table 2).

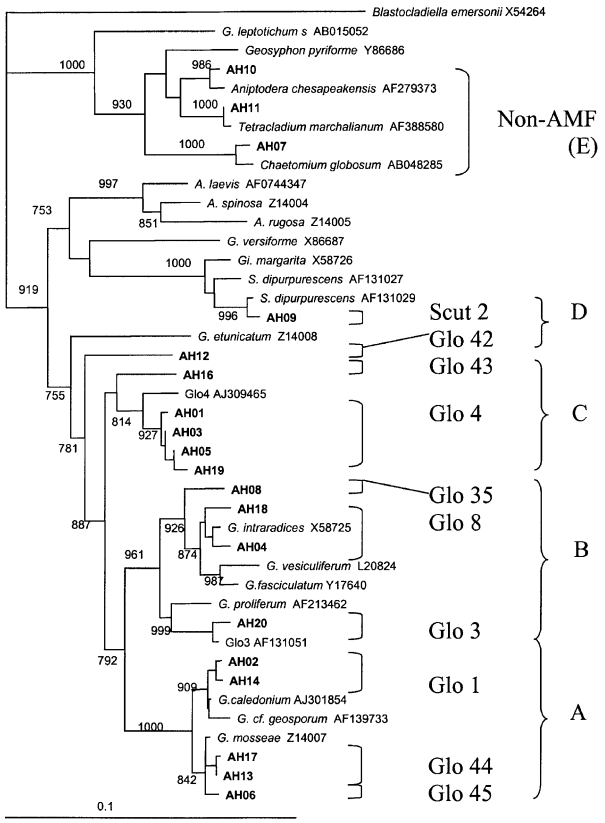

Fig. 4. Phylogenetic tree of all 19 sequence types identified for period (c) shown in bold, three of which were nonmycorrhizal fungi (non-AMF), and grouped according to neighbourhood similarities using *Blastocladiella emersonii* as an out-group and various named reference sequences (BLAST search reference numbers are given after the corresponding sequence type). All bootstrap values >75% are shown. The 10 distinct AM sequence types were named according to most similar sequences of the University of York mycorrhiza numbers. There were no closely named sequences for AH08 (Glo 35) and AH12 (Glo 42). Groups A–E refer to sequence groupings shown in Table 2.

Changes in the AM fungal community

The overall number of AM fungal sequence types (5) and clones (132) was lowest in the autumn samples and greatest in winter (8 and 200, respectively) (Table 2) and there were seasonal patterns; of the three types that were sufficiently abundant to detect patterns, Glo 1 appeared to be most abundant in summer, Glo 35 to decline progressively from the start of the experiment and Glo 4 and Glo 43 shared no obvious pattern. These grouping patterns were confirmed by MDS analysis (Fig. 5): the summer and autumn sampling times were largely distinct, but both were nested in the area of the first sampling time taken prior to treatment. Moreover, this analysis revealed no patterns corresponding to plant species in the sample, which confirmed the findings of loglinear analysis. Although all three sampling times received the same sampling effort and the same cloning technique, the amount of roots was much greater in the winter than in the autumn samples due to a combination of low birth and high root death rates during autumn (Edwards *et al*., in press), and thus differences reflect the biological reality.

The AM sequence types were re-categorized into groups A, B, C and D for loglinear analysis (Table 2). Groups A, B and C corresponded to groupings in the neighbour-joining tree (Fig. 4), but group D comprised Glo 42 and Scut 2 due to low clone numbers (Table 2). Grouping was necessary to obtain valid χ^2 values for the analysis (for a more detailed explanation of the analysis and model used, see Helgason *et al*. (1999)). However, avoiding subjective grouping, cluster analysis (data not shown) separated only Glo 4 and Glo 1 from all other AM fungal sequences. Loglinear analysis

Table 2 Total counts for arbuscular mycorrhizal (AM) and non-AM sequence types of the three sampling times during period (c) for the combined ambient and heating treatments and their grouping for loglinear analysis

	A†		B			C	D		E	
Treatment*	Glo44 and Glo45	Glo1	Glo3	Glo35	Glo8	Glo4 and Glo43	Glo42	Scut2	Non-AMF	Total
Winter sample: 26/01/01 (prior to treatment in the year-long period)										
NS‡	2	31	1	20	1	30	1	7	14	107
S/2	0	12	2	8	0	31	0	3	6	62
S	2	6	0	20	7	14	1	1	12	63
Total	4	49	3	48	8	75	2	11	32	232
Summer sample: 10/05 and 07/06/01§										
NS	2	25	0	16	0	18	7	5	12	85
S/2	4	23	6	5	0	22	1	0	14	75
S	2	13	1	5	0	14	4	5	22	66
Total	8	61	7	26	0	54	12	10	48	226
Autumn sample: 27/09 and 25/10/01§										
NS	3	16	6	0	0	28	0	0	7	60
S/2	4	8	5	3	0	20	0	0	7	47
S	1	2	12	7	0	17	0	0	2	41
Total	8	26	23	10	0	65	0	0	16	148

*Treatments were NS: no shade, S/2: 70% shade, S: 86% shade.
†AM sequence types refer to the University of York mycorrhiza numbers and fungal groups A–E are given in Fig. 4; A–D are the grouped data used for loglinear analysis in Table 3.
‡Indicates additional DNA extraction and amplification in a sample.
§Between each period no treatments were applied.

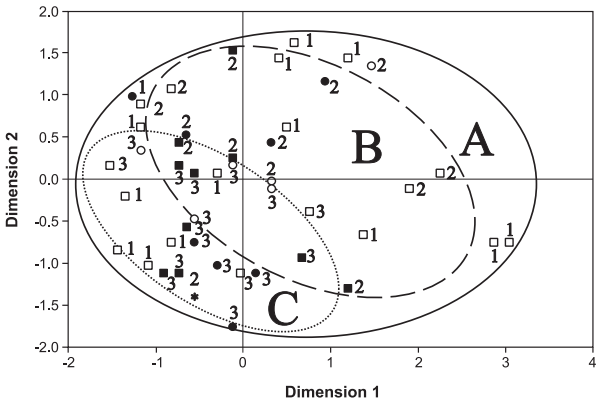

Fig. 5 Multidimensional scaling model for the three sampling times (data are given in Table 2) of the experimental period (c). The 45 data points for sampling time one (1: winter, $N = 14$), two (2: summer, $N = 15$) and three (3: autumn, $N = 16$) are encircled with A (closed line), B (dashed line) and C (dotted line), respectively; * indicates an outlier, excluded from defining individual circles (A–C). Data for 70% (S/2) and 86% (S) shade treatments were combined, giving four treatments in total: ambient temperature (squares) or soil warming (circle), combined with either unshaded (white) or shaded (black) treatments. Scaling was carried out according to frequencies for the eight AM sequence type groups (n.b. two RFLP similarities for two sequence types, see Table 2).

using all four AM groups (Table 3) revealed changes in the AM fungal community over time, which were highly significant ($P = 0.004$) and with a significant shading effect ($P = 0.025$), but no effect of soil warming; according to the cluster analysis only removal of Glo 4 and Glo 43 gave a significant ($P = 0.048$) shading effect (Table 3). Similarly, a one-way ANOVA showed a weakly significant shading effect ($F_{2,6} = 3.72$; $P = 0.089$) on the combined (Table 2) clone numbers of only the Glo 4 and Glo 43 data set.

Discussion

The most active phase of AM colonization of roots (L_RC) was during spring and summer, whereas non-AM fungi peaked in autumn; this significant negative correlation could be observed over a whole year (Fig. 2d), and has not been seen previously in a field study. AM colonization can suppress infection by root-colonizing pathogens such as *Fusarium oxysporum* in the grass *Vulpia ciliata* ssp. (Newsham *et al*., 1995). Our data suggest that such suppression occurred only early in the season. Interestingly, the overall L_RC in period (c) varied widely by about 40%, yet after 1 year returned to nearly exactly the previous year's percentage, suggesting regulation of colonization patterns of the fungal

Table 3 Results of loglinear analysis for arbuscular mycorrhizal (AM) fungal SSU rRNA sequence type groupings either according to neighbouring tree similarity or cluster analysis

	4 AM groups*			According to cluster analysis								
	All ($N = 72$)†			All ($N = 54$)			Glo 4* ($N = 36$)			Glo 1* ($N = 36$)		
Terms removed from	Chi-sq.‡	df§	*P*-value¶	Chi-sq.	df	*P*-value	Chi-sq.	df	*P*-value	Chi-sq.	df	*P*-value
Saturated model	0.0	0	1.000	0.0	0	1.000	0.0	0	1.000	0.0	0	1.000
-A*H*S*T	3.9	12	0.985	2.6	8	0.985	0.7	4	0.950	1.7	4	0.784
-A*H*S*T-A*T*H	4.8	18	0.999	8.3	12	0.763	6.5	6	0.369	3.2	6	0.784
-A*H*S*T-A*T*S	18.6	24	0.773	14.5	16	0.561	11.6	8	0.169	7.2	8	0.516
-A*H*S*T-A*H*S	16.5	18	0.560	10.4	12	0.577	3.4	6	0.763	6.7	6	0.351
-A*H*S*T-A*H*T-A*S*T-A*T	62.4	36	**	35.6	24	0.059	19.6	12	0.074	14.7	12	0.258
-A*H*S*T-A*H*S-A*H*T-A*H	17.6	27	0.915	16.3	18	0.572	8.6	9	0.474	8.2	9	0.518
-A*H*S*T-A*S*T-A*S*H-A*S	2.8	36	*	31.6	24	0.138	21.2	12	*	17.1	12	0.146

*AM sequence types as in Fig. 4; the four AM fungal groups were A–D as given in Table 2.

†Total number of counts ($N = 72$) was reduced to 54 if grouping was carried out according to cluster analysis, which separated only Glo 4 and Glo 1 (both $N = 18$, giving $N = 36$).

‡Chi-square values (Chi-sq.).

§Degrees of freedom (df).

¶Corresponding *P*-values of loglinear analysis for the saturated model and models of subsequent removal of individual factors ((A) AM sequence types (4), (H) heating (2), (S) shading (3) treatments and (T) sampling time (3)) and their interaction (for details see Helgason *et al*., 1999) with significance: $*P < 0.05$; $**P < 0.01$.

community. This colonization pattern might also indicate a seasonal impact as AM and non-AM fungal colonization probably depends on a higher proportion of young roots in spring and old or senescent roots later in the season, respectively; however, for L_RC estimates dead roots were avoided.

Shading was the most effective treatment and reduced AM L_RC in periods (a) and (c). Pot experiments have shown that under carbohydrate-limiting conditions, the plant regulates carbon transfer to the fungus (Tester *et al*., 1985; Graham *et al*., 1997), but here we show this effect for the first time under field conditions. The most likely mechanism was that shading reduced root birth (Fitter *et al*., 1998) and therefore AM L_RC, since colonization is predominantly of younger roots (Bowen, 1987). A warm winter with high sunshine levels before sampling started might have limited shade effects in period (c) because of early root growth and increased carbon content in the roots. In addition, the AM fungal community had been established for a full, undisturbed season before the spring period (c) as compared with period (a). Soil warming had less influence, but affected L_RC of AM and non-AM fungi differently: whereas it reduced AM L_RC (Fig. 2b), non-AM L_RC was increased (Fig. 2c). This might largely reflect impacts of soil warming on root dynamics and below-ground respiration: first, by increasing root death (Norby & Jackson, 2000) and second, by increasing root respiration and thus increasing root turnover; the increased amount of old root biomass would be easily invaded by root pathogens or saprotrophic fungi. Whereas root respiration seems to acclimate (Atkin *et al*., 2000), we have only assumptions about AM fungal respiration (Cooper, 1984; Smith & Read, 1997). These inferences must be qualified by an acknowledgement that in this study only the roots were warmed. Had shoots been warmed (e.g. by use of infrared lamps), a different result might have been obtained. No study has yet combined root and shoot warming in the field to simulate properly the impacts of a warmer climate.

Stepwise regression of L_RC data supported our initial hypothesis that light is more influential on AM L_RC than temperature (Table 2); whereas higher but relatively short-term PAR sums were associated with higher AM L_RC, relatively long and warmer temperature periods increased non-AM L_RC. Further, increasing PAR sums generally increased L_RC_{arb} indicating increased metabolic activity (Wilson & Tommerup, 1992; Blee & Anderson, 1998). In our study, the light period during the 3–7 days prior to sampling can be hypothesized as the most influential environmental variable and explained 8–19% of the variation in AM L_RC. These findings are consistent with fast carbohydrate transport to the AM fungi, also detected in a natural ectomycorrhizal community by girdling trees

(Högberg *et al*., 2001). AM fungi also seem to rely on recently fixed carbon (Jakobsen & Rosendahl, 1990; Andersen & Rygiewicz, 1991; Wright *et al*., 1998b).

In contrast, higher mean soil temperature mostly decreased AM L_RC but increased non-AM L_RC in the summer and autumn period (c); whereas warmer air temperature might increase photosynthesis and therefore carbon allocation to AM fungi, constant soil warming would increase carbon demand by roots (Fitter *et al*., 1999) without affecting photosynthesis, thus reducing available carbon to the AM fungus. However, weather conditions might also have played a crucial role in determining soil warming effects. The exceptionally warm winter before period (c) resulted in already high AM L_RC and limited a possible spring soil warming impact.

We detected only ten AM fungal sequence types (species), a low figure compared with Bever *et al*. (2001) and Fitter's (2001) survey of published data. Our finding of mostly *Glomus* spp. and in particular the absence of any *Acaulospora* species from this former horticultural site, corresponds to the low AM species diversity detected in other arable sites (Daniell *et al*., 2001), and attributed to soil disturbance destroying the ERM (Giovannetti & Gianinazzi-Pearson, 1994; Helgason *et al*., 1998).

AM fungal clone numbers do not necessarily correlate with percentage L_RC, nor are the most abundant AM fungi in the roots necessarily ecologically important symbionts (Helgason *et al*., 1999); the benefit provided to the plant might differ among fungal species (Bever *et al*., 2001) as well as the extent and spatio-temporal distribution of the ERM (Sylvia, 1990). However, in this study, as also in Helgason *et al*. (1999), the ease of amplification and cloning reflected measured AM L_RC in the paired root samples fairly well. Failure of amplification does not necessarily mean that a fungal type is not present, but rather reflects either inadequate DNA or absence of roots of a particular host type in the sample; this shortfall can only be overcome by unrealistically large sample numbers. Only shading was a significant influence with both approaches; although fungal growth responses to temperature are commonly reported for pot experiments, we did not detect any soil warming impacts with the sampling techniques used here. A major difference between field and pot experiments on mycorrhizas (Fitter *et al*., 2000) is that in field studies a well-established plant community exhibits root turnover and the fungal community maintains an existing mycelium; in pot experiments with seedlings the root system is developing and the fungal symbiont is establishing a mycelium. In our experimental design, the community had fully exploited the upper 20 cm of soil by the first spring period, as monitored by root growth dynamics (Edwards *et al*., in press), and roots showed stable colonization patterns by the time of the year-long period. The evidence here therefore suggests that soil temperature does have a little impact on mycorrhizal colonization. However, we measured root colonization; impacts on the ERM remain unknown, and the only other study investigating soil warming impacts on AM fungal growth (Monz *et al*., 1994) did not address treatment impacts on plant growth.

Merryweather & Fitter (1998b) detected seasonal changes in the AM fungal community in bluebell roots, especially for the winter-active fungus *Scutellospora dipurpurescens*. In our study, the only clear seasonal effect was increased abundance of Glo 1 in summer. Further, Scut 2 was only found in winter (11 clones) and summer (10 clones), but the overall numbers were small. Shading decreased clone numbers and hence presumably L_RC of one particular AM fungus, Glo 4 (Table 3), and its decline coincided with lower abundance of *T. repens* under shade. Our results are of particular interest as there is growing awareness of niche separation in AM fungal communities (Bever *et al*., 2001), and of the possibility of AM host specificity (van der Heijden *et al*., 1998; Helgason *et al*., 2002). However, there are only indications for spatio-temporal separation, which in some cases could be due to winter activity (Clapp *et al*., 1995; Merryweather & Fitter, 1998b; Helgason *et al*., 1999). Niche separation might also be driven by light availability determining carbon allocation to the AM fungal community, and the use of available carbohydrates by the fungi either for reproduction or growth (Helgason *et al*., 1999). These considerations must be viewed in the light of confusion over the current systematics of AM fungi and in particular the species concept for AM fungi. We have combined AM sequence types for analysis without any *a priori* data on the ecological affinities of the types, but until better links are made between sequence types and function, no alternative approach is available.

Although most effects on AM intra-radical colonization observed in this study probably reflected impacts on plant growth, direct responses by AM fungi, for example to temperature, are possible and responses might be species specific; future studies have to address these questions. We did not test for effects on the ERM, yet environmental effects on growth and turnover of the ERM might determine whether soils will function as carbon sink or sources under a changing climate (Fitter *et al*., 2000).

Our findings suggest that, first, the extent of AM colonization is predictable and regulated, although the overall AM fungal community in a native grassland may display seasonal dynamics. Second, available PAR

rather than small changes in soil temperature control colonization by AM fungi; hence, the results correspond to findings about root dynamics (Aguirrezabal *et al*., 1994; Fitter *et al*., 1998, 1999). Third, AM and non-AM fungi interact in ways that are potentially significant for the plant community, and are susceptible to climate change. Finally, molecular data showed that individual AM fungi may react uniquely to any changes in climate under field conditions. Seasonal changes in this low-diversity AM fungal community indicate niche separation and, for the first time we have shown that shading can alter the field abundance of a particular AM fungal species, possibly through an impact on a preferred plant host species. It is clear that further research investigating any impacts of climate change on ecosystem functioning must not only incorporate their natural AM fungal communities but should also focus on niche separation and community dynamics of AM fungi. Whether AM fungi respond directly to environmental changes remain to be answered in future work.

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