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Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland

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Summary

1 The arbuscular mycorrhizal (AM) fungi colonizing plants at a woodland site in North Yorkshire (UK) have been characterized from the roots of five plant species (*Rubus fruticosus* agg. L., *Epilobium angustifolium* L., *Acer pseudoplatanus* L., *Ajuga reptans* L. and *Glechoma hederacea* L.), and identified using small-subunit rRNA (SSUrRNA) gene amplification and sequencing.

2 Interactions between five plant species from the site and four co-occurring glomalean fungi were investigated in artificial one-to-one AM symbioses. Three of the fungi were isolated from the site; the fourth was a culture genetically similar to a taxon found at the site. Phosphorus uptake and growth responses were compared with non-mycorrhizal controls.

3 Individual fungi colonized each plant with different spatial distribution and intensity. Some did not colonize at all, indicating incompatibility under the conditions used in the experiments.

4 *Glomus hoi* consistently occupied a large proportion of root systems and outperformed the other fungi, improving P uptake and enhancing the growth of four out of the five plant species. Only *G. hoi* colonized and increased P uptake in *Acer pseudoplatanus*, the host plant with which it associates almost exclusively under field conditions. Colonization of all plant species by *Scutellospora dipurpurescens* was sparse, and beneficial to only one of the host plants (*Teucrium scorodonia*). *Archaeospora trappei* and *Glomus* sp. UY1225 had variable effects on the host plants, conferring a range of P uptake and growth benefits on *Lysimachia nummularia* and *T. scorodonia*, increasing P uptake whilst not affecting biomass in *Ajuga reptans* and *Glechoma hederacea*, and failing to form mycorrhizas with *A. pseudoplatanus*.

5 These experimental mycorrhizas show that root colonization, symbiont compatibility and plant performance vary with each fungus–plant combination, even when the plants and fungi naturally co-exist.

6 We provide evidence of physical and functional selectivity in AM. The small number of described AM fungal species (154) has been ascribed to their supposed lack of host specificity, but if the selectivity we have observed is the general rule, then we may predict that many more, probably hard-to-culture glomalean species await discovery, or that members of species as currently perceived may be physiologically or functionally distinct.

Key-words: *Archaeospora*, *Glomus*, phosphorus, plant growth, *Scutellospora*, sequence diversity, SSUrDNA

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Introduction

Fungi in the order Glomales form arbuscular mycorrhizas (AM) with the majority of land plants, but are among the least well understood of the soil biota. There is an urgent need to study these organisms, not only attempting to discover how many species there are, but also characterizing the role these symbioses play in ecosystem functioning (Tilman *et al*. 1996; Read 1998). Fewer than 200 species of these globally important fungi have been described (Morton & Benny 1990), and it has been presumed that their specificity of symbiotic association must be low in view of the vastly more numerous plant species (perhaps 200 000) with which they form mycorrhizas (Smith & Read 1997). New glomalean taxa are continually being discovered, but there is no indication that they will prove to be as numerous as the plant species they colonize, unless many lower taxonomic levels or physiologically distinct 'types' are identified. Although specificity appears to be low in some species studied, others may be compatible only with a narrow range of host plants (Ravnskov & Jakobsen 1995).

The roots of only a small fraction of the world's plants have been examined, and it is impossible to say with accuracy what proportion of plant species are mycorrhizal. However, estimates based on numerous, disparate samplings suggest that values over 90% may be reasonable, and that two-thirds of all species form arbuscular mycorrhizas (Fitter & Moyersoen 1996). Physiological studies suggest that many plants may rely almost wholly on mycorrhizal fungal assistance for phosphate acquisition (Merryweather & Fitter 1995a; Streitwolf-Engel *et al*. 1997), and that disease and pest resistance (Newsham *et al*. 1994; Gange & Bower 1997; West 1997) and drought resistance (Ebel *et al*. 1996) may also be conferred on plants. The structure and dynamics of plant communities may be heavily dependent on mycorrhizal fungi (van der Heijden *et al*. 1998a). The AM fungi are all obligately biotrophic, depending on green plants to supply the carbon compounds essential for tissue production and survival (Ho & Trappe 1973), and their populations are thus entirely dependent on the formation of mycorrhizas. It has also been reported that the maintenance of diverse plant communities may be highly dependent on mycorrhizal diversity (van der Heijden *et al*. 1998a, 1998b), which would not be expected if the specificity of association between AM fungi and their hosts was universally low.

Our AM research has concentrated on a single semi-natural woodland site in North Yorkshire. Early work involved a single plant species, *Hyacinthoides non-scripta* (Liliaceae), a vernal geophyte which is highly mycorrhizal and wholly dependent on AM for its phosphate supply (Merryweather & Fitter 1995a, 1996). Arbuscular mycorrhizas also play an important role in the life history of this obligately mycorrhizal plant. Glomalean fungi invade the seedling's single root, and colonization intensity increases as plants age and bulbs descend through the soil profile to soil horizons where P is highly deficient and mycorrhiza are essential (Merryweather & Fitter 1995b). The diversity of AM fungi associated with *H. non-scripta* is high (Helgason *et al*. 1999). During the growing season the roots of adult plants are colonized by a succession of eight or more AM fungi which include one species of *Scutellospora* and several representatives of the genera *Acaulospora* and *Glomus* (Merryweather & Fitter 1998a, 1998b; Helgason *et al*. 1999).

Natural communities of plants and their symbiotic glomalean fungi are interdependent, yet we do not know: (i) what determines which AM fungal species occur in which natural communities; (ii) what determines the pattern of colonization of the plants by the fungi in any community; (iii) what are the consequences for each partner of particular symbiotic associations; and (iv) the impact that AM symbioses have on the structure and dynamics of those communities. What little has been discovered suggests that natural ecosystems are complex mycorrhizal communities of interactive above- and below-ground biota (Read 1998). The soil contains networks of fungi that interconnect the roots of separate individual plants (Chiarello *et al*. 1982; Newman 1988; Watkins *et al*. 1996). It is probable that numerous fungi interact with individual plants, all or selected individuals of a species, or large parts of a community, conferring a range of benefits at a variety of taxonomic and physiological levels.

We carried out parallel field and laboratory studies in order to answer three questions. Are there measurable functional differences among AM fungal isolates trapped from the same field site? Do fungal isolates induce differential benefit in host plants typical of the field site? Do these patterns of colonization and host benefit reflect patterns observed in the field? First, we sampled five plant species typical of the Pretty Wood plant community, and characterized the AM fungal sequence types present using the AM1 primer to select for AM fungi (Helgason *et al*. 1998). The range of variation in the field AM fungi was compared with isolates that had previously been trapped from the same site. Second, in order to examine the relationships of representatives of both plants and AM fungi at our field site, we synthesized one-to-one fungus–plant pairs in order to measure (i) the degree of affinity of plants and AM fungi as shown by root colonization; and (ii) the physiological impact of each fungus–plant combination as reflected by plant P uptake and growth. We grew plants at a range of P addition rates to enable us to compare the increased P uptake that might be stimulated by AM fungal colonization with that achievable by non-symbiotic plants.

Our specific objectives in this study were to determine (i) the diversity of AM fungi present in host plants other than *H. non-scripta* that are typical of the field site; (ii) whether trapped isolates are present in plant roots in the field; (iii) whether all fungi colonized all

plants equally; and (iv) whether the consequences of colonization were dependent on the fungus or on the symbiosis.

Methods

LABORATORY TESTS: EXPERIMENTAL DESIGN

The glasshouse experiment was conducted during summer 1998 using 1 : 1 plant–fungus combinations in plant pots. The plants and AM fungi used represented possible mycorrhizal associations at our field site at Pretty Wood, North Yorkshire, UK (National Grid ref. SE 732867, altitude 50 m).

There were five fungal treatments: 1. Non-mycorrhizal control; 2. *Scutellospora dipurpurescens* Morton & Koske (isolate WV109A); 3. *Glomus hoi* Berch & Trappe (isolate UY110); 4. *Archaeospora trappei* Morton & Redecker (isolate UY1245); and 5. *Glomus* sp. (isolate UY1225) which does not resemble any described species (C. Walker, personal communication). The three isolates prefixed UY were obtained by trap culture from Pretty Wood soil. Despite repeated attempts using fresh spores from trap culture, we have failed to isolate *S. dipurpurescens*, which has been shown by both morphological and molecular evidence to be an important component of the glomalean fungal assemblage at Pretty Wood (Merryweather & Fitter 1998a,1998b,1998c; Helgason *et al*. 1999). Therefore equivalent germplasm of *S. dipurpurescens* was obtained from the International Vesicular–Arbuscular Mycorrhiza collection (INVAM, University of West Virginia, USA). No differences have been detected between field-collected specimens of this fungus and the INVAM culture by either morphological (Merryweather 1997; Merryweather & Fitter 1998a) or molecular techniques (Helgason *et al*. 1998). All inoculum was generated in large pots containing a sand and Terra-Green (calcined attapulgite clay; Oil Dri UK Ltd, Wisbech, Cambridgeshire, UK) mix in which one individual of each experimental species was planted. Five species were used in the experiment: *Acer pseudoplatanus* L. (sycamore; Aceraceae); *Ajuga reptans* L. (bugle; Lamiaceae); *Lysimachia nummularia* L. (creeping jenny; Primulaceae); *Glechoma hederacea* L. (ground ivy; Lamiaceae); and *Teucrium scorodonia* L. (wood sage; Lamiaceae). Plants will be referred to by their generic names.

Plants of *Acer* were grown from seed in pots for 30 weeks. The other four plant species were grown from spring shoot cuttings dipped in hormone rooting powder and planted in individual compartments in Plantpak multi-well trays containing Terra-Green. They were maintained in the glasshouse under propagator tops until well rooted (6–12 weeks). Experimental plants were transferred to 13 cm diameter plastic pots containing 1100 g of a growing medium consisting of four parts heat-sterilized (48 h at 100 °C) dried silica sand ('Leighton Buzzard Sand' grade 16/30; Hepworth Minerals & Chemicals, Sandbach, Cheshire, UK) and one part autoclaved woodland soil (from the Ah horizon in which water-extractable P concentration = 1.24 μg g^{-1} measured after autoclaving; Merryweather & Fitter 1996), to which was added 25 g AM fungal inoculum (equivalent to 2.25% w.w., and consisting of chopped and mixed substratum, roots, fungal hyphae and spores from a fresh, vigorously sporulating pot culture).

Phosphorus amendments equivalent to 0, 1, 3, 9 and 27 μg P per g growth medium were given as bonemeal (GEM, Oswaldtwistle, Lancashire, UK) mixed into the growing medium. Bonemeal was used as a slowly available form of P that could be dispersed through the soil, increasing the potential need for AM assistance in plant P assimilation. *Acer* received the full range of bonemeal treatments. A pilot experiment in which non-mycorrhizal plants were grown with P treatments as above established that *Ajuga*, *Glechoma*, *Lysimachia* and *Teucrium* are able to assimilate P, even in the absence of mycorrhizas, from bonemeal applied at between 3 and 27 μg g^{-1}. Therefore in the main experiment those species received a restricted range of bonemeal treatments at rates equivalent to 0, 3 and 27 μg g^{-1} P.

Each plant–fungus–P treatment was replicated five times, and pots were randomized and maintained on an open wire-mesh bench in the glasshouse. Timing of different sections of the experiment was dependent on the availability of plants and fungal inoculum. The herbaceous species were all stoloniferous, and the plant material used was new (clonal) stolons initiated in spring or early summer. Consequently the experiment was conducted in sections initiated when material became available. The separate sections of the experiment were: 1. *Acer* (all fungi; initiated 8 April 1998); 2. *Ajuga* and *Glechoma* (all fungi; 6 May 1998); 3. *Teucrium* (except *S. dipurpurescens*; 6 May 1998); 4. *Lysimachia* (all fungi; 1 July 1998) and *Teucrium* (*S. dipurpurescens* only; 1 July 1998).

Plants were harvested for assay when well grown, but before they were pot-bound: *Lysimachia*, 9 weeks; *Ajuga*, *Glechoma* and *Teucrium*, 12 weeks; *Acer* 16 weeks.

MEASUREMENTS

Plants were removed from pots, washed and divided into their component parts before drying (70 °C, 3 days), weighing and P analysis. All species were separated into leaves, stems (*Glechoma*: stolons; *Ajuga*: petioles) and roots. A pre-weighed sample of root was taken for staining and data adjusted accordingly. The magnified intersection method of McGonigle *et al*. (1990) using 100 intersections was used to quantify root colonization by AM fungi, expressed as percentage of root length colonized (%RLC).

Roots were stained with acid fuchsin using a phenol-free modification of the method of Kormanik & McGraw (1982), mounted on microscope slides, and examined with a Nikon Optiphot-2 compound

microscope. Under most circumstances mycorrhizas were visible under bright-field illumination, but when AM fungi were poorly stained or invisible using this method, epifluorescence illumination (Merryweather & Fitter 1991) provided a greatly enhanced image, especially in the case of *A. trappei*.

Plant fresh weights were measured at the beginning and end of the experiment, and fresh and dry weights of separate plant parts (48 h at 70 °C) at the end.

Phosphorus concentration, [P], of leaf, root and stem was measured on samples of *c*. 200 mg dried material by the molybdenum blue method (Allen 1974) after acid digestion. Phosphorus content of plant parts was calculated as dry weight × [P].

STATISTICAL METHODS

Statistical analysis was performed using SPSS version 7.1. The effects of the different fungi, added P treatments, and the interaction between the two were tested using the general factorial (bivariate) general linear model. Where added P had no effect on measured responses (all plants except *Acer*), data from the range of added P treatments were combined. Differences between mean values were tested *post hoc* with Bonferroni's modified LSD multiple-range test ($P < 0.05$).

FIELD SURVEY: SAMPLE COLLECTION

Four sites were sampled in July 1996 within an area of 0.06 km^2 centred on Ordnance Survey grid reference SE730692. An *Acer pseudoplatanus* L. and a *Quercus petraea* Leib. canopy were identified in the eastern and western parts of this area, and two sampling sites were located under each, separated by 100–200 m. Up to three species of host plant were sampled in each site, representing the dominant species present. The understorey vegetation beneath each canopy was very different, resulting in different species being sampled from each canopy type. Beneath the *Quercus* canopy, *Rubus fruticosus* agg. L. (bramble; Rosaceae) and *Epilobium angustifolium* L. (rosebay willowherb; Onagraceae) were sampled, and from beneath the *Acer* canopy, *Ajuga reptans*, *Glechoma hederacea* and *Acer* itself were sampled (Table 1). Five plants of each host species were randomly selected and removed from each site. For each understorey plant, the entire root system of each plant was sampled; for *Acer*, root samples were obtained by removing soil blocks close to the base of *Acer* saplings. The roots were thoroughly washed and a subsample of 0.5–1 g was frozen prior to DNA extraction. DNA extracts from some root samples consistently failed to yield PCR products, probably due to low colonization. Dried roots from the experiment described above were stored in a cool, dry place prior to DNA extraction.

Spores were sampled from the stock cultures used to generate the inoculum for the laboratory experiment described above. Spores were obtained from pots by mixing a sample obtained using a 0.5 cm diameter

Table 1 Number of clones of each RFLP type present in each host plant

Canopy	Host plant	Sample size	Acau1	Acau2	Acau4	Acau5	Glo2 + 7*	Glo3	Glo5	Glo8	Glo9	Scut1	*Acaulospora*	*Glomus*	*Scutellospora*	Total
Acer	*Glechoma*	9	23		1	–	3	6		5	–	–	24	14	–	38
	Ajuga	7	16	1	–	–	7	1	1	–	–	–	17	9	–	26
	Acer	6	2	–	–	–	6	–	–	3	9	–	2	18	–	20
Subtotal			41	1	1	–	16	7	1	8	9	–	43	41	–	84
Quercus	*Epilobium*	5	5	–	–	–	–	–	–	–	–	5	5	–	5	10
	Rubus	10	13	–	1	1	1	–	–	–	1	5	15	2	5	22
Subtotal			18	–	1	1	1	–	–	–	1	10	20	2	10	32
Grand total			59	1	2	1	17	7	1	8	10	10	63	43	10	116

Samples from replicate sites within each canopy type have been combined for this analysis. Note that RFLP types Glo2 and Glo7 have been amalgamated for the analysis; see text for explanation.

corer with water, allowing large particles to settle, and sieving through a 32 or 60 μm mesh sieve.

MOLECULAR ANALYSIS

DNA was extracted from plant roots using a potassium ethyl xanthate method (Edwards *et al*. 1997), and from spores by crushing with a sterile pipette tip in 20 μl TE buffer. Where necessary, samples were diluted and reconcentrated using a 100 kDa microfilter (Microcon, Millipore UK, Watford, UK). Fungus-specific PCR, cloning, sequencing and analysis was carried out as described by Helgason *et al*. (1999). Partial small-subunit (SSU) DNA fragments (*c*. 550 base pairs) were amplified using *Pfu* DNA polymerase (Stratagene, LaJolla, CA, USA) with a universal eukaryotic primer NS31 (Simon *et al*. 1992) and AM1 (Helgason *et al*. 1998). The reaction was performed in the presence of 0.2 mM dNTPs, 10 pmoles of each primer and the manufacturer's reaction buffer. PCR was carried out for 30 cycles (10 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min; 19 cycles at 95 °C for 30 s, 58 °C for 1 min and 72 °C for 3 min; and 1 cycle at 95 °C for 30 s, 58 °C for 1 min and 72 °C for 10 min) on a Gradient96 Robocycler (Stratagene). The resulting blunt-ended products were cloned into pCR-Script Amp SK(+) or the *Sma*I site of pBluescript SK(+) (Stratagene) and transformed into *Escherichia coli* (XL1-blue MRF′ or JM109). Putative positive transformants were selected and screened using standard T3/T7 amplification. Up to 10 positives from each individual were digested with the restriction enzymes *Alu*I and *Hinf*I, according to the manufacturer's instructions (Promega, Madison WI, USA). PCR products were re-amplified from selected clones using T3/T7, cleaned using Qiaquick PCR purification spin columns (Qiagen Ltd, Crawley, UK) and sequenced on an ABI 377 automated sequencer according to the manufacturer's instructions using the Dye terminator cycle sequencing kit with AmpliTaqFS DNA polymerase (ABI Perkin-Elmer, Warrington, UK) using T3 and T7 as the sequencing primers.

DATA ANALYSIS

Forward and reverse sequences were aligned using Autoassembler (ABI Perkin-Elmer) or Lasergene SeqMan (DNAStar Inc., Madison WI, USA). CLUSTALX (Thompson *et al*. 1997) was used for multiple alignment and neighbour-joining phylogeny (Saitou & Nei 1987), using *Blastocladiella emersonii*, a chytrid, as the outgroup. Statistical analysis was performed using SPSS version 8.0 and ARLEQUIN (Schneider *et al*. 2000).

Results

LABORATORY TEST: ROOT COLONIZATION

Roots of control plants did not become colonized by AM fungi. Other, non-AM fungi were occasionally seen in control- and fungus-treated plants. There was no colonization in *Acer* inoculated with *A. trappei*, *Glomus* sp. UY1225 or *S. dipurpurescens* (Fig. 1a), nor in *Glechoma* inoculated with *S. dipurpurescens* (Fig. 1c). Among the other fungus–plant pairings there were four individual cases of plants with zero colonization: *Ajuga* (2) and *Lysimachia* (1) inoculated with *S. dipurpurescens*, and *Ajuga* (1) inoculated with *Glomus* sp. UY1225.

The intensity of colonization varied according to both fungus and plant (Fig. 1). In all plant species, the most intense colonization was by *G. hoi* and least by *S. dipurpurescens*. Virtually entire root systems were colonized by *G. hoi*, and large quantities of spore-bearing external hyphae remained attached to washed and stained roots. Root colonization by *A. trappei* and *Glomus* sp. UY1225 was patchy, and external hyphae were not visible in quantity on roots. Root systems were patchily but locally intensely colonized by these fungi. The two *Glomus* species bore numerous intraradical vesicles. Colonization of roots by *S. dipurpurescens* occurred in very limited regions of the roots, and stained roots frequently retained a tuft of external hyphae bearing the auxiliary cells characteristic of the Gigasporaceae.

PLANT RESPONSES TO ADDED P

There were very few effects of P addition. *Acer* showed the expected response, with increased biomass and P content (Fig. 2) but no consistent changes in P concentration. When grown with *S. dipurpurescens*, both *Glechoma* and *Teucrium* had increased biomass at the highest P addition (27 p.p.m.) relative to the non-mycorrhizal controls (Fig. 3).

PLANT RESPONSES TO INOCULATION WITH AM FUNGI

The functioning of each plant–fungus pair was examined by plotting the increase in P content against the increase in biomass of each plant, both relative to the mean for the uninoculated controls for that plant (means for 0, 3 and 27 p.p.m. P additions). A null response was estimated by plotting a line whose slope was the mean P concentration (P content/biomass) of the controls and therefore represented growth at that constant concentration (Fig. 4). If changes in both P content and biomass resulting from fungal treatment are exactly matched, then points will fall along this line. Where points fall above this line, P content has increased more than biomass, and there has been an increase in P concentration. Where points can be fitted by a regression line, the slope of the line represents the P concentration of the increased growth, i.e. $(p_m - p_{mn})/(B_m - B_{nm})$ where B = biomass.

Acer pseudoplatanus

Glomus hoi markedly increased both plant P content and growth (Fig. 4a). Although none of the other fungal species visibly colonized the roots, and although

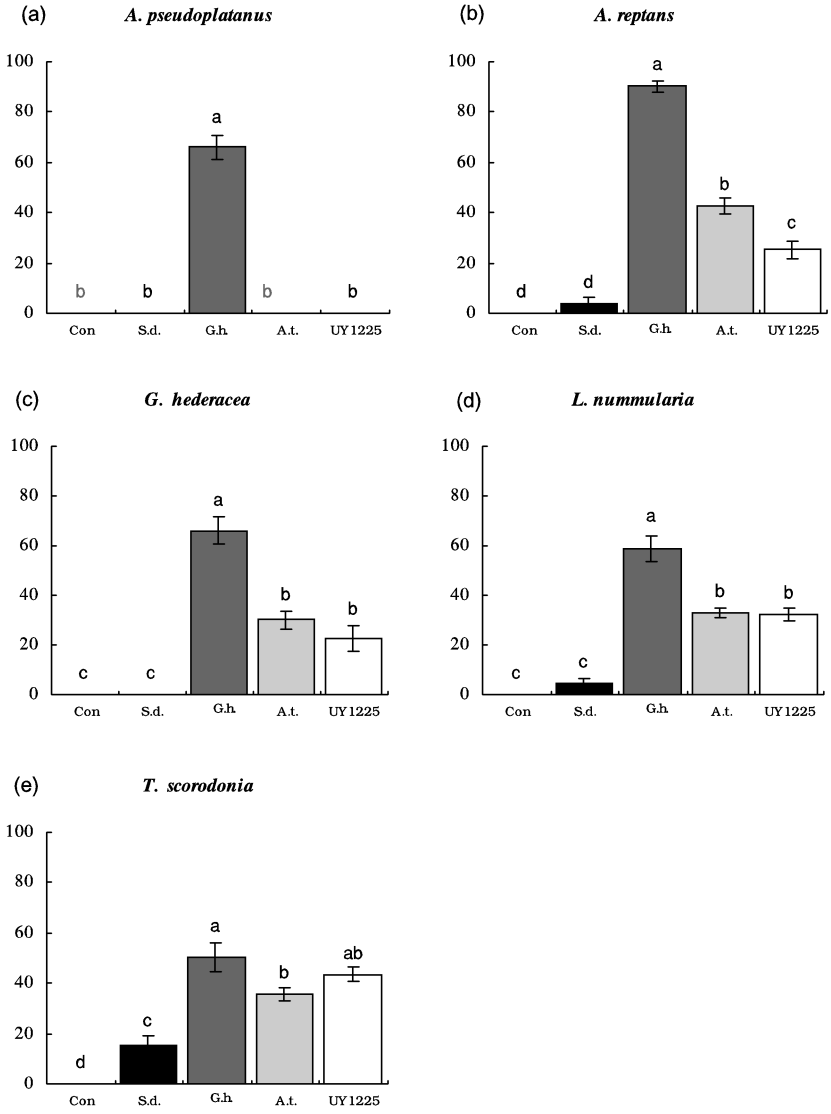

Fig. 1 Percentage of root length colonized by AM fungi (%RLC) in five plant species. Con, non-mycorrhizal control; (S.d. = *Scutellospora dipurpurescens*; G.h. = *Glomus hoi*; A.t. = *Archaeospora trappei*; UY1225 = *Glomus* sp. UY1225). Data from 0, 3 and 27 p.p.m. Phosporus-addition treatments were pooled. Bars are standard errors. Letters label significantly different mean values ($P < 0.05$ by the Bonferroni modified LSD test).

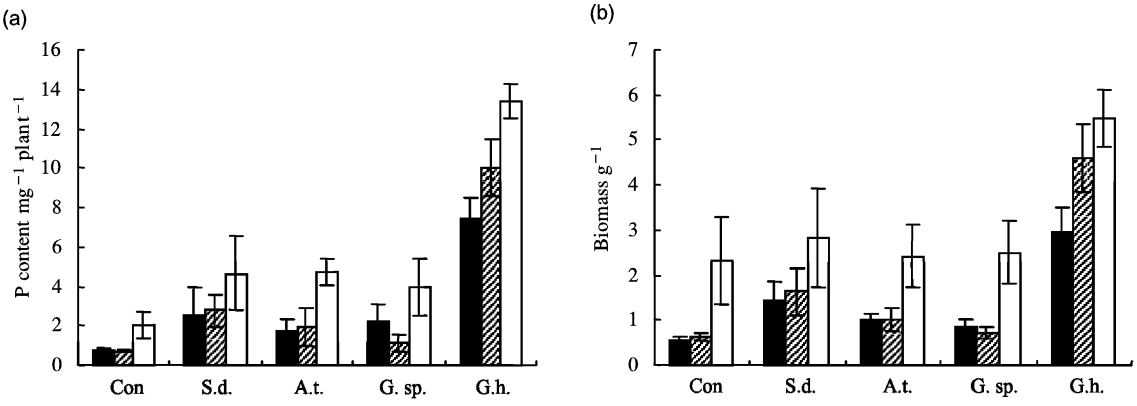

Fig. 2 Responses of *Acer pseudoplatanus* in terms of (a) P content and (b) biomass to inoculation by AM fungi. Con, non-mycorrhizal control; (S.d., *Scutellospora dipurpurescens*; G.h., *Glomus hoi*; A.t., *Archaeospora trappei*; G. sp., *Glomus* sp. UY1225). Solid columns are nil added P; hatched columns 3 p.p.m. added P; open columns 27 p.p.m. added P. Data for 1 and 9 p.p.m. P additions are omitted for clarity. Bars are standard errors. Letters label significantly different mean values within each added P group ($P < 0.05$ by Bonferroni's modified LSD test).

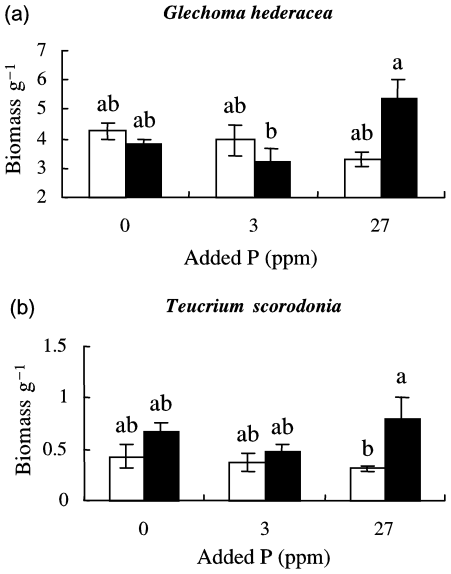

Fig. 3 Responses of plants colonized by *Scutellospora dipurpurescens* to added P: (a) *Glechoma* (colonized at very low level); (b) *Teucrium* (white columns, non-mycorrhizal control; black columns, *S. dipurpurescens*). Bars are standard errors.

they had no significant effect on P content, the P concentration of the plants fell on the same trajectory as for those colonized by *G. hoi*.

Ajuga reptans

Scutellospora dipurpurescens was the only fungus to increase *Ajuga* biomass, although it did not increase plant P content significantly and plants colonized by it fell on the same concentration trajectory as the controls (Fig. 4b). In contrast, plants colonized by the other three fungi all had elevated tissue P concentration and total P content, but showed no growth response.

Glechoma hederacea

There was no response to the very low level of colonization by *S. dipurpurescens* (Fig. 4c). The increase in P uptake and lack of growth response with *G. hoi* was similar to that seen in *Ajuga*; consequently there was a large increase in P concentration. Although both

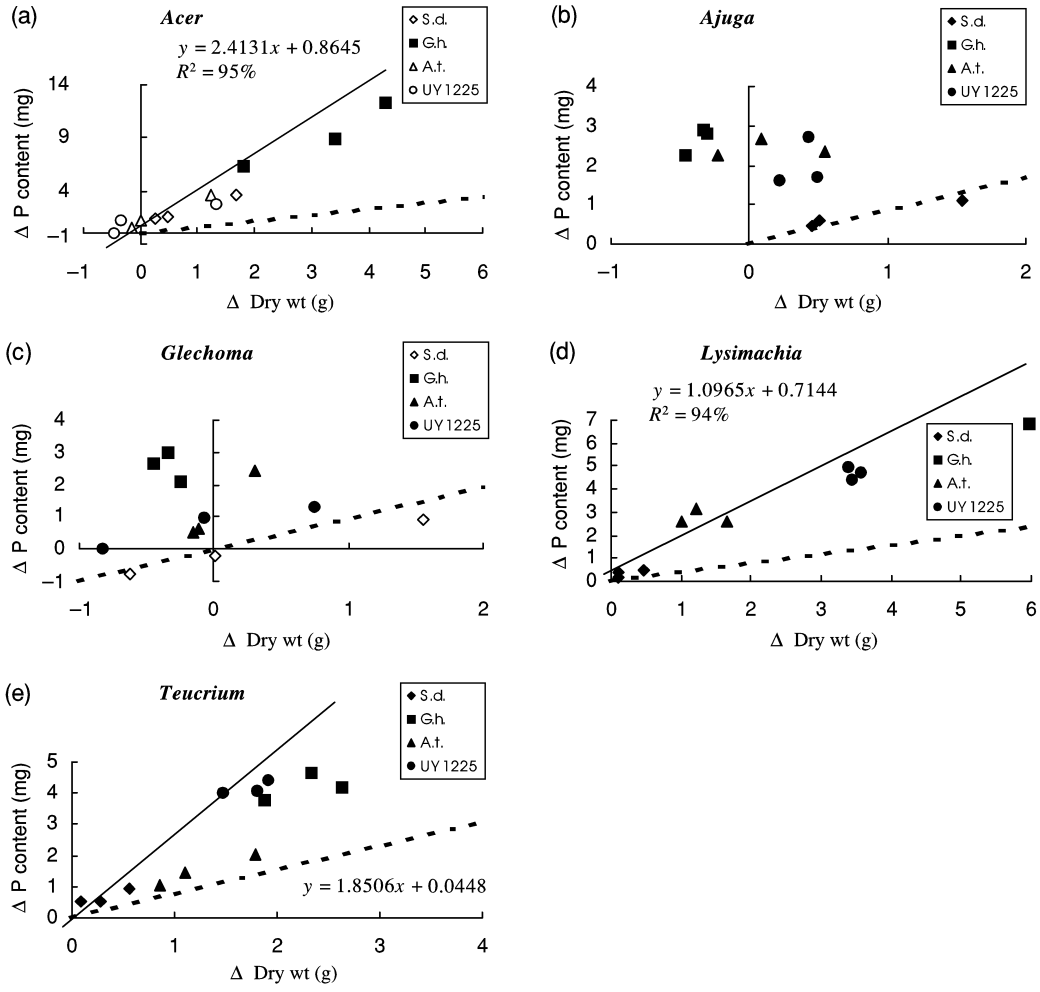

Fig. 4 Change (Δ) in dry weight plotted against change in P content of five plant species, each inoculated with (but not necessarily colonized by) four AM fungi. Con, non-mycorrhizal control; (S.d. = *Scutellospora dipurpurescens*; G.h. = *Glomus hoi*; A.t. = *Archaeospora trappei*; UY1225 = *Glomus* sp. UY1225). Solid lines are linear regressions, shown where significant, through four separate data sets in each chart (regression data are shown). Broken lines represent data from non-mycorrhizal controls using a slope derived from the mean P concentration.

A. trappei and *Glomus* sp. UY1225 increased tissue P concentration, P content was not significantly increased and there was no growth response.

Lysimachia nummularia

All fungi except *S. dipurpurescens* greatly improved P uptake and biomass, the most effective being *G. hoi* (Fig. 4d). Only plants colonized by *A. trappei* were flowering at the time of harvest.

Teucrium scorodonia

Phosphorus content and biomass increased with all four fungi (Fig. 4e), although only marginally with *S. dipurpurescens*, and all except plants colonized by *A. trappei* contained elevated tissue P concentration.

FIELD SURVEY: TAXONOMIC IDENTITY OF THE SEQUENCES

In all, 66 clones derived from the mycorrhizal plant roots were sequenced. The great majority of the sequences (60 clones) fell within the Glomales SSUrRNA clade (Fig. 5). Three of the remainder matched the SSUrRNA sequence of other fungi: two pyrenomycetes and one basidiomycete. The others were unrelated to SSUrRNA, were usually of a slightly different length to the fungal products, and probably represent mispriming during the PCR reaction (see Helgason *et al*. 1999 for detailed discussion).

Six of the eight sequence groups previously found in *Hyacinthoides* (Helgason *et al*. 1999) were also present in the other species sampled at the same time. Glo3, previously found in *Hyacinthoides* and present in both *Glechoma* and *Ajuga*, matches UY1225 (*Glomus* sp.). An additional five sequence types not found in *Hyacinthoides* were found. Two of these matched cultures that have been isolated from Pretty Wood soil. Glo9 is most closely related to the pot culture UY110 (*G. hoi*), though it is not identical to it, and Acau2 matches UY1198 (*Acaulospora* sp.). Of the remainder, two each matched published sequences with >97% similarity: Glo8 matched *G. intraradices* (GenBank Accession no. X58725), and Glo5, *G. etunicatum* (Z14008) (Fig. 5). Glo7, while clearly *Glomus*, was not closely related to sequences from any named strains. This brings to 13 the number of sequence groups found at this site.

Using the sequence information, a further 56 clones were classified as AM fungal sequences according to *Hinf*I/*Alu*I RFLP type, a total of 116 clones from the five host-plant species (Table 1). In most cases, sequences with the same RFLP type cluster together. Glo3 and Scut1 groups each contain two RFLP types, neither of which occurs in other sequence groups. In both cases the RFLPs are classed as a single group because the two RFLP types do not form separate clades (Fig. 5). Acau1 is a single RFLP type group that consists of two separate clusters (A and B in Fig. 5). Glo2 sequences have three RFLP types. An *Ajuga* (Aj1.5, Fig. 5) and *Acer* (Ac3.7) clone have the same RFLP type (Glo7), but the latter groups with Glo2, and the two groups have therefore been merged. Two clones from *Acer* that also group with Glo2 have a third, unique RFLP type. It is likely that these groups will be further subdivided as information becomes available. Clones obtained from spores of a *Glomus* sp. pot culture, UY1227, share the common Glo2 RFLP, but are related to the Glo8 clade. However, 19 of the 25 Glo2 clones in this and our previous study were sequenced, so it is unlikely that the remainder have been misclassified.

DISTRIBUTION OF FUNGAL TAXA AMONG HOST PLANTS

In order to compare the frequencies of the sequence types, clone numbers were bulked by AM family. The data in Table 1 suggest that the AM families are not distributed homogeneously among the five host-plant species. *Scutellospora* was not found in *Acer*, *Ajuga* or *Glechoma*; *Glomus* was rare or absent from *Rubus* and *Epilobium*; and *Acaulospora* was only found twice (10%) in *Acer* despite being the most frequently sequenced genus. We can test for random association among host plants and AM fungi using an extension of Fisher's exact test (Schneider *et al*. 2000). This test uses a Markov chain to generate a distribution of possible contingency tables, and assigns a probability to the observed data. In this case the probability was calculated to be nearly zero ($P < 0.00001$), suggesting that it is almost impossible that these data could have arisen by chance from a randomly distributed population of AM fungi. The plant–fungus combinations that are significantly over-represented are *Acer*/*Glomus* ($P < 0.01$), *Rubus*/*Scutellospora* ($P = 0.01$) and *Epliobium*/*Scutellospora* ($P < 0.01$); those significantly under-represented are *Glechoma*/*Scutellospora* ($P = 0.02$), *Acer*/*Acaulospora* ($P < 0.01$), *Rubus*/*Glomus* ($P < 0.01$) and *Epilobium*/*Glomus* ($P = 0.01$) (Table 1).

In our previous study of *Hyacinthoides* at this site, we showed that the frequency of sequence types differs with canopy type and site (Helgason *et al*. 1999). In this study, where *Hyacinthoides* was present beneath both canopy types, *Glomus* species were more frequent beneath the *Acer* canopy, while *Acaulospora* species were more frequent beneath the *Quercus* canopy. The present study reflects the general pattern shown in *Hyacinthoides* roots. There was a significant difference between *Quercus* and *Acer* sites when the combined data from all host plants were analysed in this way ($\chi^2 = 38.7$, 2 d.f., $P < 0.0001$). The pattern of change, however, appears to be different from that for *Hyacinthoides* alone. In that host plant, *Scutellospora* clones were rare in July but were spread evenly among sites, *Glomus* clones were rare under the *Quercus* canopy, and *Acaulospora*

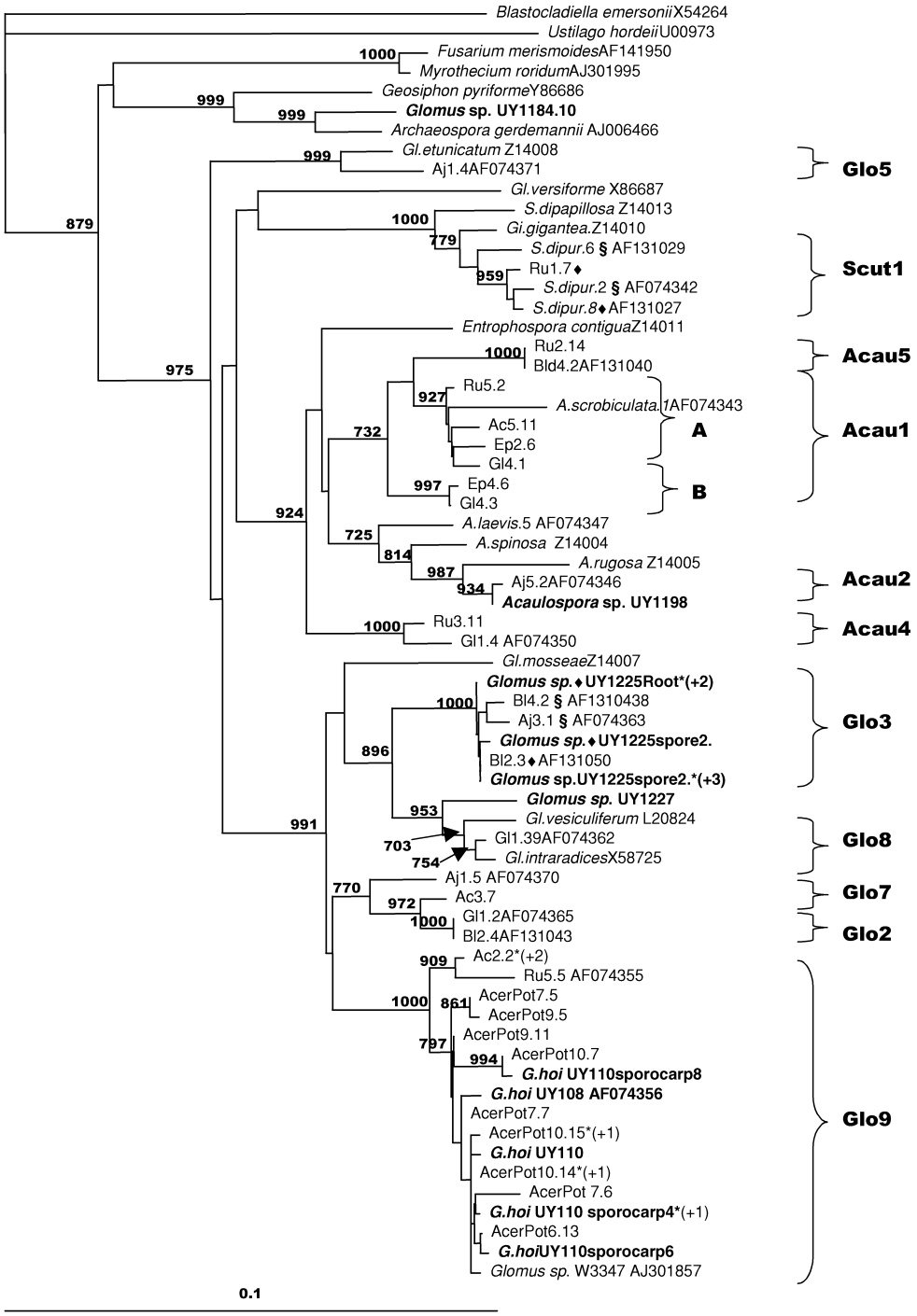

Fig. 5 Neighbour-joining tree showing representatives of all sequence types identified in host plant roots, using *Blastocladiella emersonii* (Chytridomycota) as the outgroup. The sequence types (Glo5, etc.; Table 1) identify groups whose sequences share the same RFLP type. All bootstrap values >70% are shown (1000 replicates). Individual field clones are identified by host plant (Aj = *Ajuga*; Ep = *Epilobium*; Gl = *Glechoma*; Ru = *Rubus*; Ac = *Acer*), root sample number and clone identifier. Samples with code BL (bluebell, *Hyacinthoides non-scripta*) were determined by Helgason *et al*. (1999) from this site. AcerPot = sequences identified from *Acer* roots taken from the laboratory experiment. Named glomalean sequences (in italics) are from Simon (1996); Gehrig *et al*. (1996); Redecker *et al*. (2000); Schuessler *et al*. (2001); Helgason *et al*. (1998, 1999); those in bold are isolates from Pretty Wood soil. Sequence type identifiers are consistent with those previously published by Helgason *et al*. (1998, 1999). *, Additional multiple identical clones of the number indicated have been sequenced. All new sequences have been submitted to the GenBank database (Accession nos AF485861–AF485890). ◆ and § identify two RFLP variants within sequence groups Scut1 and Glo3. The division of Acau1 is discussed in the text.

clones were rare beneath the *Acer* canopy. By contrast, the change in frequency of *Acaulospora* in this study was not as marked, with a small reduction of *Acaulospora* clones from 63 to 50%, and *Scutellospora* under the *Quercus* canopy represented 31% of the clones. As was the case for the *Hyacinthoides* data, *Glomus* clones were rare under the *Quercus* compared to the *Acer* canopy (Table 1).

Discussion

PRETTY WOOD ISOLATES AND THEIR DISTRIBUTION IN THE FIELD

We have shown that a wide range of AM fungal genotypes colonize plants at Pretty Wood. This study is among the first to demonstrate the non-random distribution of AM fungi among different host plants *in planta* in the field. We show that within a relatively diverse plant community, the distribution of the AM fungi is significantly non-random. This complements the data of Merryweather & Fitter (1998b), who conclude on the basis of root colonization and spore production that there are major and minor contributors to the fungus–plant symbiosis between AM fungi and *Hyacinthoides* in Pretty Wood. This is not in itself sufficient evidence for us to infer host preference due to functional selectivity, but it demonstrates that factors operate in the environment to make some host plant–fungus combinations more likely than others.

We have examined plant responses to AM colonization by single fungi in mycorrhizas which could occur at our field site. Such one-to-one mycorrhizas in artificial conditions are unlikely to be typical of those found in natural communities, but they are an important preliminary component of our 'dissection' of mycorrhizal functioning in the field. Only a few experimental studies of mycorrhizal functioning have examined the behaviour of the natural cohabitants of an ecosystem (Streitwolf-Engel *et al*. 1997; van der Heijden *et al*. 1998a,1998b; Brundrett *et al*. 1999), and in none of these was the distribution of the fungi in the field known. Most other studies have used ecologically unrelated AM fungi cultures and crop plants.

Three of the four fungi used were from our trap-culture programme. *Glomus* sp. UY1225 has occurred in several trap-culture attempts, and has been found in many plant species in Pretty Wood including *Ajuga* (from whose roots it was originally isolated), *Glechoma* and *Hyacinthoides*, but only rarely from *Acer*. Sequences from spores and colonized roots of the isolate *Glomus* sp. UY1225 closely match Glo3 sequence types (Fig. 5). This is one of the common sequence types in understorey plants at Pretty Wood but, unlike the other common sequence groups identified at the site, e.g. Glo2 and Acau1, it is also amenable to trap culture.

We have also frequently isolated *G. hoi* in trap culture, but only from soil beneath young *Acer* and the roots or rhizosphere soil of *Mercurialis perennis* growing beneath *Acer*. In the field, the only sequences identified were of the type shown in Fig. 5. While they are the most closely related sequences to *G. hoi*, they are not identical. As the isolate was trapped from Pretty Wood soil, it must be present as a viable organism with inoculum potential, but it appears to be a rare component of the colonizing fungi. The field variant has been identified only by molecular methods in roots of *Acer* and of *Rubus*, another woody plant. A total of 10 clones from field roots, 31 clones from UY110-inoculated *Acer* plants from the experiment, and five clones from spores and roots of the isolate were screened with additional restriction digest analysis (data not shown), and no overlap was identified. However, the similarity of the two sequence types is within the range of similarity (>97% identified within individual subcultures of *S. dipurpurescens* WV109A (S.dipur2 & S.dipur6 in Fig. 5), so they might reasonably be considered to originate from a single 'species' aggregate.

In common with other workers who have found *Scutellospora* species to be difficult (e.g. Brundrett *et al*. 1999), we have been unsuccessful in our attempts (189) to culture *S. dipurpurescens* from Pretty Wood, and had to use an imported isolate in the experiment. Although near-identical to *S. dipurpurescens* at Pretty Wood in terms of its SSUrDNA sequence and morphology, it is physiologically distinct at least in culturability, and therefore may not have behaved identically.

Archaeospora trappei is a highly invasive fungus and is easily isolated in trap culture. It has been found in Pretty Wood soil spore assemblages. We now know that the molecular method used here would not amplify members of the Archaeosporaceae that have already been sequenced (Redecker *et al*. 2000) so, unsurprisingly, this taxon has not been identified in roots, nor have we been able to characterize this isolate. Thus the AM cultures we had available for experimentation were numerous individual isolates of the few species that are amenable to trap culture, three of which were probably representative of the field community, and one whose ecological affiliations are unknown at present. *Glomus* species tend to dominate trap-culture attempts (Brundrett *et al*. 1999), and most of our isolates are of fungi in this genus.

HOST PLANT–FUNGUS RESPONSES

Colonization varied between fungi and between plants. *Glomus hoi* occupied a large proportion of root systems, whereas other fungi colonized partially and patchily. There were four cases of failure to colonize. Incompatibility may have been the cause of failure to colonize *Acer* by *A. trappei* and *Glomus* sp. UY1225, which are usually very aggressive fungi under culture conditions and successfully colonized all other plant species. Similarly, the failure of *S. dipurpurescens* to colonize roots of *Acer* and *Glechoma* may be due to incompatibility, but our experience suggests that *S. dipurpurescens* colonizes *H. non-scripta* more efficiently if present in soil as an established mycelium (Merryweather & Fitter 1998c).

The plots of increased P content against increased biomass relative to controls (Fig. 4) reveal the nutrient-use efficiency (NUE; Aerts & Chapin 2000; Koide *et al*. 2000). Strictly, NUE_P is given by dW/dP (the inverse of the slope in Fig. 4); we have chosen to present the data as, in effect, dP/dW as the slope then directly displays the change in tissue P concentration. Where

Table 2 Responses of five experimental plants to four arbuscular mycorrhizal fungi

Fungus	Plant	Leaf [P]	Root [P]	Other [P]	Biomass	P Cont.
Scutellospora dipurpurescens	*Acer*	–	–	–	–	–
	Ajuga	–	–	–	↑	–
	Glechoma	–	–	–	–	–
	Lysimachia	↑	–	–	–	–
	Teucrium	–	↑	↑	↑	↑
Archaeospora trappei	*Acer*	–	–	–	–	–
	Ajuga	↑	↑	↑	–	↑
	Glechoma	–	↑	↑	–	–
	Lysimachia	↑	↑	↑	↑	↑
	Teucrium	–	–	–	↑	↑
Glomus hoi	*Acer*	↑	↑	↑	↑	↑
	Ajuga	↑	↑	↑	–	↑
	Glechoma	↑	↑	↑	–	↑
	Lysimachia	↑	↑	↑	↑	↑
	Teucrium	↑	↑	↑	↑	↑
Glomus sp. UY1225	*Acer*	–	–	–	–	–
	Ajuga	↑	↑	–	–	↑
	Glechoma	–	↑	–	–	–
	Lysimachia	↑	↑	↑	↑	↑
	Teucrium	↑	↑	↑	↑	↑

↑ = Significant increase when compared with controls. Data from added P treatments are combined, except *Acer* in which data for 0 p.p.m. added P are given.
'Other' in *Acer*, *Lysimachia* and *Teucrium* = stem; in *Ajuga* = petioles; in *Glechoma* = stolons.

a common line fits a set of points, the plant is maintaining a constant P concentration and therefore constant NUE_P. These plots reveal whether mycorrhizal colonization had a uniform impact on plant growth and P nutrition.

On this basis, there were four main types of plant response to fungi (Table 2; Fig. 4):
(a) roots uncolonized and no growth response: NUE_P as in control plants (e.g. *Glechoma* with *S. dipurpurescens*)
(b) roots uncolonized, NUE_P reduced (i.e. higher tissue P concentration), seen in *Acer* with all fungi except *G. hoi*
(c) roots colonized, growth stimulated, but NUE_P reduced and P concentration increased; this occurred in *Acer* with *G. hoi*, in *Lysimachia* with all fungi except possibly *S. dipurpurescens*, and in *Teucrium* with all fungi except possibly *A. trappei*
(d) roots colonized, P content increased with no change in biomass, resulting in high tissue P concentration and low NUE_P.
This pattern occurs in *Glechoma* and *Ajuga* with all fungi except *S. dipurpurescens*.

Of these patterns, (a) is the expected response of an uncolonized plant and (b) may be artefactual. The fact that the points for all fungi with *Acer* fall on the same NUE_P trajectory suggests that there may have been cryptic colonization, either in a form that did not stain effectively or else at too low a frequency to be detected. On the other hand, sequences of *G. hoi* have been found almost exclusively in *Acer* roots at the field site (see below).

Pattern (c) is the classical mycorrhizal growth response (Mosse 1957; Koide *et al.* 2000), with increased growth but proportionally greater increase in P content leading to increased tissue P concentration and reduced NUE_P. Mycorrhizal plants have been shown selectively to allocate P to reproduction (Koide *et al.* 1988; Bryla & Koide 1990; Merryweather & Fitter 1996), and Wright *et al.* (2000) proposed that early flowering in *Holcus lanatus* was linked to high shoot P concentration. In this experiment, early flowering was recorded only in *Lysimachia* colonized by *A. trappei*, but this effect was not linked to high P concentration, at least when measured.

Pattern (d) occurred only in those plants with a stoloniferous growth habit (*Glechoma* and *Ajuga*). *Teucrium* is also stoloniferous, and it is possible to interpret the data for that species (Fig. 4e) in the same way. In these species, P is stored during the main part of the season and used during the period of stolon production in spring. Stolon growth was not initiated during this experiment.

RELATIONSHIP BETWEEN FUNCTION AND FIELD DISTRIBUTION

Glomus sp. UY1225 appears to be a 'typical' AM fungus, showing a relatively broad host range, although it is more frequent in the sites where *Acer* occurs. It has been found colonizing a single *Acer* root, but not one sampled for this study (data not shown). Its distribution could be due either to selection for the host-plant species found beneath the *Acer* canopy, or to features of the soil environment beneath the *Acer* canopy. Functionally, this isolate also shows no clear benefit to a particular host plant, but some benefit to most of them. The one plant species it did not colonize extensively in

the pot was *Acer*, the only species in the field survey from which it was absent.

Glomus hoi UY110 presents a less clear-cut association with a field sequence where the field-derived sequences are the most closely related to, but distinct from, the isolate. Although these are genetically closely related isolates, they may be unrelated functionally and represent two separate 'species'. At the other extreme, the variation present in the UY110 isolate might represent a fraction of the polymorphism within a single, variable species that includes the field sequences. In this scenario, additional sampling in the field would yield a wider variety of Glo9 sequences from *Acer* roots, and the variation within UY110 has been restricted by trapping: it is a founder population. Both variants are present in the field population as viable inoculum associated with *Acer*, and only the difficulty of sampling field roots prevented us from making better estimates of the extent of genetic variation present in field samples. The fact remains that the Glo9 sequence type has mostly been identified in *Acer*, suggesting that both variants are associated with *Acer*, an unexpected restriction in distribution for two functionally unrelated taxa. That, taken with the profound impact of *G. hoi* UY110 on *Acer* growth in the experiment, suggests that this may be a genuine case of selectivity.

Until recently (Ravnskov & Jakobsen 1995; van der Heijden *et al.* 1998b; this study) there has been little evidence for selectivity between particular arbuscular mycorrhizal fungi and host plants (Smith & Read 1997). The failure of *A. trappei* and *Glomus* sp. UY1225, which colonized all other species, to colonize roots of *Acer* presents a case for mycorrhizal selectivity. This is enhanced by the fact that *G. hoi*, which in the field has been found almost exclusively in roots of *Acer*, was the only fungus in this experiment to benefit *Acer*, and that this was the only plant species that *Glomus* sp. UY1225 failed to colonize either in the pot or in the field. However, points for the three non-colonizing fungi fall on the same P/biomass trajectory as points for *G. hoi* (Fig. 4), and it is possible that colonization was successful, but at such a low level that it was not detected (*Acer* roots are difficult to clear and internal colonization might not always be seen).

Occurrences of *G. hoi* sequences in field roots of plants other than *Acer* are extremely rare. We suggest that in field soils and roots there is a diverse community of AM fungi whose component taxa play particular roles in association with individual hosts. In this system *G. hoi* forms mycorrhizas preferentially with *Acer*. We now have evidence from both molecular (Helgason *et al.* 1999) and morphological (Merryweather & Fitter 1998b) studies that *Hyacinthoides* is more likely to be colonized by *Glomus* spp. (although not by *G. hoi*) when growing under *Acer* than when under *Quercus*. Under both canopies *S. dipurpurescens* is the dominant fungus in mycorrhizas in the early part of the *Hyacinthoides* growing season. It declines in abundance 5–6 months after initial colonization, and its place is taken by *Acaulospora* species under the *Quercus* canopy. These taxa are not abundant where young *Acer* forms the canopy (Merryweather & Fitter 1998b). Colonization by *Glomus* spp. here may be a case of invasion by an aggressive opportunist, and might be a parasitism of *Hyacinthoides*. When competition from other fungi is removed, as in this experiment, *G. hoi* may invade the roots of plant species within which it is normally unable to compete with fungi present, even enhancing P uptake which would probably not occur in the field.

The evidence provided here shows that the highly selective patterns of association between plants and fungi found in roots at our field site are reflected in the functioning of their mycorrhizas. The close physical and functional association of *Acer pseudoplatanus* and *G. hoi* is the first example of functional selectivity to be observed in this symbiosis. In the field, *G. hoi* colonizes *Acer* roots almost exclusively. In culture it was the only species capable of colonizing *Acer* roots and the only species to promote P uptake and growth in *Acer*.

It is generally assumed that AM fungi are non-specific (Smith & Read 1997). The principal evidence for this is that (i) most cultured AM fungi are non-specific; and (ii) only 150 or so AM fungal species are described, even though the Glomales colonize perhaps 200 000 plant species. It is likely that the non-selectivity of cultured fungi accurately reflects their broad host range and their consequent ease of culture. The many taxa that appear to be hard or impossible to culture may turn out to be much more selective. It is also likely that there are many more than 150 AM fungal species. The single temperate field site we have studied, which has a flora of <100 plant species (<0.05% of the described species in the world) is home to at least 13 AM fungal taxa (nearly 10% of described species). Such mismatches are the norm (Fitter 2001), and probably reflect large undescribed diversity in the Glomales. It is likely that numerous cases will be discovered where particular plant–fungus combinations are favoured in AM; whether these are due to genetic factors that favour particular associations or co-existence resulting from the spatial and temporal pattern of occurrence of the two partners in the soil remains to be discovered. Speculation as to why AM show so much less specificity than ectomycorrhizas (e.g. Hoeksema 1999) is therefore probably premature.

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