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Abstract Arbuscular mycorrhizal (AM) symbioses may alter the competitive abilities of plant species and facilitate positive interactions such as nutrient transfer between plants. They are therefore particularly interesting components in agroforestry systems. We studied spatial variation of AM colonisation on a cut-and-carry fodder production site (agroforestry plot) of the legume tree *Gliricidia sepium* and the fodder grass *Dichanthium aristatum*. Roots of the two plant species were sampled under the tree canopies and on the adjacent grass plot at 1 and 3.5 m from the first tree row where *G. sepium* roots also occur. Roots of *D. aristatum* were also sampled on a nearby grass monocrop. Colonisation of arbuscules, vesicles and hyphae in root samples was visually determined, and AM fungal species were identified by DNA sequencing. Colonisation and frequency of types of AM formations varied statistically significantly between the species and sampling points. Arbuscular colonisation in *G. sepium* roots was higher under the tree canopies than on the adjacent grass plot. Soil nutrient content, particularly P and N, and interspecies competition are the most probable explanations for the observed variation in AM colonisation. Both arbuscular colonisation and arbuscule:vesicle ratio in *D. aristatum* roots was lower on the *D. aristatum* monocrop than on the agroforestry plot under or near the tree canopies. Intercropping could stimulate AM symbiosis in *D. aristatum*. Both plant species formed symbiosis with *Rhizophagus intraradices*, indicating potential for interplant N transfer via common mycelial networks of AM-forming fungi.

Keywords Arbuscules · Vesicles · Root colonisation · *Rhizophagus intraradices* · Vertisol · DNA sequencing

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Introduction

Communities of fungi which form arbuscular mycorrhizae (AM) are recognised as important components of material cycles in natural and agricultural ecosystems. An increasing number of studies indicate how AM fungal species and communities interact not only with individual host plants but with entire plant

communities, affecting their composition and structure in various ways. Nutrient acquisition by AM-forming fungi may increase when plants are grown in mixtures of species as compared to monoculture (Hodge 2003). Symbiosis with AM-forming fungi may change competitive abilities of plant species, favouring either the already dominant or the less competitive species depending on their mycorrhizal dependency (Scheublin et al. 2007). Nutrients may be transferred between plant species via common mycelial networks of AM-forming fungi (He et al. 2003), and potential for direct N transfer from legumes to associated crops has received considerable interest in intercropping systems as a means of improving crop nutrition and reducing competition for soil N. Trees in agroforestry systems may also act as reservoirs of AM-forming fungi between cropping seasons, and facilitate AM colonisation and establishment of annual crops (Ingleby et al. 2007). Overall, AM symbioses are capable of altering competitive relationships between plants and mediating their coexistence, thereby contributing to diversity, productivity and stability of the plant communities as a whole (Hart et al. 2003; van der Heijden et al. 1998).

Variation in the occurrence of fungal structural formations arbuscules, vesicles or hyphae may reveal functional differences in the AM symbiosis between plant species or growth conditions. Arbuscules, highly branched structures which are formed when the AM fungal hyphae penetrate individual cells in the root cortex, are known as the sites where carbon and nutrients are transferred between the plant and the fungal symbiont (Smith and Read 2008). Highest N and P concentrations in plant tissue coincided with highest arbuscular colonisation in temperate grasslands where P limited plant growth (Garcia and Mendoza 2008). Transfer of N from a legume tree to an associated grass via common mycelial networks of AM-forming fungi correlated positively with the extent of arbuscular colonisation in the legume roots (Jalonen et al. 2009). Two different types of arbuscules, *Arum*-type and *Paris*-type, are recognised. Their occurrence has been associated with fitness of the fungal and plant symbionts, while the functional significance of the differences remains unclear (Dickson et al. 2007). Vesicles occur in the intra- or intercellular space and are assumed to be storage organs for energy reserves within the fungus (Smith and Read 2008). Increased vesicle colonisation within plant roots has been observed at N-limited sites, which could reflect higher investment of carbohydrates from the plant to the fungus in order to maintain nutrient supply under sub-optimal conditions (Treseder and Allen 2002). Some studies have also compared ratio of arbuscules to vesicles, suggesting it as an indicator of the relative cost or benefit of the fungus to the host plant (Braunberger et al. 1991; Titus and Leps 2000). In contrast, total AM colonisation is often dominated by hyphal colonisation and may be unaffected, e.g. by interspecific competition or nutrient limitation, even when they result in increases in mycorrhizal dependency or vesicle colonisation (cf. Scheublin et al. 2007; Treseder and Allen 2002).

Common mycelial networks are formed between individual plants when the extraradical hyphae of their AM fungal symbionts fuse, i.e. form anastomosis. Anastomoses have empirically been demonstrated only between fungal isolates which are genetically identical or belong to the same population (Giovannetti et al. 2006; Croll et al. 2009; see also Mikkelsen et al. 2008). Shared mycorrhizal symbionts would, thus, be a precondition for transfer of N between plant species via common mycelial networks of AM-forming fungi.

Because of the functional importance of AM symbiosis, its spatial variation is of interest in intercropping systems where spacing and densities of plant species may be managed to optimise productivity or sustainability of the system (Hamel 1996; Boddington and Dodd 2000; Shukla et al. 2009). Analysis of changes in AM fungal communities and colonisation patterns can help to interpret observed changes in plant communities, and adjust management practices to facilitate favourable interactions between plant species.

Strong evidence of direct N transfer between plant species has been obtained on a well-studied cut-and-carry fodder production plot of the legume tree *Gliricidia sepium* (Jacq.) Kunth ex Walp and the fodder grass *Dichanthium aristatum* (Poir) C.E. Hubbard in Guadeloupe, Lesser Antilles. Nitrogen isotopic signatures of the N recipient *D. aristatum* correlated with isotopic signature of *G. sepium* roots but not with the surrounding soil (Sierra and Nygren 2006). The grass was observed to preferentially absorb N released from the tree as opposed to soil N sources (Daudin and Sierra 2008). It was subsequently shown under semi-controlled conditions that at least part of the N transfer

occurs via common mycelial networks of AM-forming fungi (Jalonen et al. 2009).

In this study we analysed spatial variation of AM colonisation on the *G. sepium–D. aristatum* plot (agroforestry plot in the following). Arbuscular mycorrhizal colonisation in plant roots was measured along a gradient of soil nutrient content and fine roots of the legume tree, at increasing distances from the tree rows. Characteristics of the plant community along the gradient gradually change from tree-dominated to grass-dominated. Our specific objectives were to (i) analyse how AM formations in *G. sepium* and *D. aristatum* roots vary with the characteristics of the plant community on the agroforestry plot, and in comparison to a *D. aristatum* monocrop, and (ii) determine whether *G. sepium* and *D. aristatum* form symbiosis with same AM fungal species as a precondition for hyphal anastomoses and N transfer via common mycelial networks. We compare the results with results of plant interactions and interplant N transfer from previous studies at the plot, to discuss what they imply about plant competitive or mutualistic relationships and N transfer via common mycelial networks of AM-forming fungi.

Material and methods

Study site

The study was conducted at the Godet Experimental Station of the French National Institute for Agronomic Research (INRA) in Guadeloupe, French Antilles (16°25′ N, 61°30′ W, 10 m a.s.l.). The climate in the area is warm and sub-humid, annual mean air temperature being 26 °C and annual mean rainfall 1300 mm. Dry season lasts from February to July, and 30 % of the annual rainfall occurs during that time. The soil on the site is Vertisol with 80 % of clay rich in smectite, developed over coral reef limestone and with pH of 7.8. Average soil depth is 0.5 m. Organic carbon content of the soil is 33.1 g kg^{-1}, organic N content 3.1 g kg^{-1}, and mineral N content varies from 10 to 20 mg kg^{-1} depending on the season (Sierra et al. 2007). About 95–98 % of the soil mineral N is in the form of NH_4^+ (Sierra et al. 2002).

The agroforestry plot was established in the area in 1989 by planting cuttings of *G. sepium* in natural grassland of *D. aristatum*. The trees were planted in North–South aligned rows at spacing of 0.3 × 2 m. Each of these tree-grass plots were 20 × 13 m in size and separated from the others by grass plots without trees, 15 m of width. At the time of this experiment the actual tree density of the tree-grass plots was about 12 000 ha^{-1} because of mortality. The plots were not trenched. Therefore, *D. aristatum* on the adjacent grass plots is in contact with *G. sepium* roots, but not with the tree canopies.

The site was managed since its establishment according to a cut-and-carry practice, with partial tree pruning every 2–6 months and grass cutting every 40–50 d. All cut material was removed from the site. Last pruning before the sampling was in December 2005. Phosphorus and K fertilisers were applied five times since the establishment of the site at 100 kg [P] ha^{-1} and 150 kg [K] ha^{-1}, last time in 2003. Available soil P is lower on the tree-grass plot than on the adjacent grass plots (11.5 vs. 18.1 mg kg^{-1}; Dulormne 2001), probably because of large biomass exports as tree prunings. Soil organic N content decreases with distance from the tree rows (Table 1). Fine root densities of the two plant species correlate negatively, with root density of *G. sepium* decreasing and that of *D. aristatum* increasing with distance from the tree rows (Table 1). Competition between the tree and the grass for soil nutrients extends to approximately 3 m from the tree rows (Daudin and Sierra 2008), although tree roots are found in the whole grass plot.

A *D. aristatum* monocrop located approximately 200 m from the agroforestry plot was selected for comparison. Soil on the monocrop is similar to that on the agroforestry plot prior to tree planting, with organic C content of approximately 21.7 g kg^{-1} and total N content 2.1 g kg^{-1} (Dulormne et al. 2003). Grass was managed by cutting every 40–50 d as on the agroforestry plot. As opposed to the agroforestry plot, *D. aristatum* on the monocrop is in contact neither with *G. sepium* canopies nor roots.

A number of studies have been conducted on the agroforestry plot since its establishment, including effects of shading of the tree canopies on the growth of *D. aristatum* (Cruz 1997); effects of the pruning regimes on N_2 fixation, biomass allocation and phenology in *G. sepium* (Nygren and Cruz 1998; Nygren et al. 2000); dynamics of competition and complementarity between the two plant species for nutrients and water (Dulormne 2001; Dulormne et al.

Table 1 Soil total N content and fine root densities of *Gliricidia sepium* and *Dichanthium aristatum* at the agroforestry plot (Sierra and Nygren 2006)

Distance from the first *G. sepium* row	Soil total N (g kg^{-1})	Fine root density (g dm^{-3})	
		G. sepium	*D. aristatum*
−1 m[a]	3.05	0.43	1.52
1 m	2.92	0.29	2.38
4 m	2.81	0.22	3.14

[a] Between the two first tree rows

2004); and N transfer in field conditions and the factors affecting it (Sierra and Nygren 2006; Daudin and Sierra 2008). For detailed descriptions of the site and the soil, see Daudin and Sierra (2008), Dulormne et al. (2003), and Sierra et al. (2002).

Sampling

Root samples of *G. sepium* and *D. aristatum* were collected on the agroforestry plot and the *D. aristatum* monocrop in August 2006. Roots were sampled along four transects: on the tree-grass plot between the first two tree rows (T1), on the adjacent grass plot 1 m from the first tree row (T2) and approximately 3.5 m from the first tree row (T3), as well as on the *D. aristatum* monocrop (T4; *D. aristatum* roots only; Fig. 1). In light of plant biomass and root density gradients of the two species (Table 1); (Sierra and Nygren 2006; Daudin and Sierra 2008), *G. sepium* dominates on the tree-grass plot (T1) and *D. aristatum* at 3.5 m away from the rows on the grass plot (T3), whereas radical competition is most even at 1 m away from the tree rows (T2). To evaluate the possibility of shared arbuscular mycorrhizal symbionts between *G. sepium* and *D. aristatum*, samples were taken at points where roots of both species were present. As the density of tree roots decreased drastically with distance from the tree rows, tree roots could not always be located at the exact spot at 3.5 m from the rows (T3). Search for roots was started at 3.5 m distance and radially expanded until tree roots were located. Grass roots were then sampled at the same point. Average distance from the tree row for these samples was 3.55 ± 0.14 m. On each transect six replications at 1 m spacing were collected for each plant species, except on T4 where samples were taken at 2 m spacing from a N–S aligned row. Total number of samples was 18 for *G. sepium* and 24 for *D. aristatum*.

All root samples were collected at a depth of 0–10 cm with surrounding soil and stored at +6 °C until processed later on the same day. Roots were then washed clean of soil. In order to determine mycorrhizal colonisation within plant roots, 12 × 10 mm fragments of fine roots per location and species were sampled close to the root tips and conserved in 60 % ethanol for further analysis. Additional 6 × 10 mm fragments were sampled per location and species for sequencing mycorrhizal fungal DNA, and stored at −70 °C.

Visual determination of AM colonisation

The sampled root fragments were stained following the method of Phillips and Hayman (1970). The roots were cleared at 2.5 % KOH in 85 °C water bath for 30 min and rinsed with distilled water. They were then stained in a lactoglycerol mixture (lactic acid, glycerol, and water 1:1:1) with 0.05 % w/v Trypan Blue, in 85 °C water bath for 25 min. The samples were immersed in lactoglycerol without the stain to remove excess colour, changing the solution daily until it remained clear. The samples where then conserved in a mixture of 50 % of glycerol and 50 % of water until mounting.

Mycorrhizal colonisation in the roots was visually determined applying the magnified intersections method (McGonigle et al. 1990). Briefly, the roots were mounted directly from the glycerol-water mixture on microscope slides, and examined with 400× magnification at 150 cross sections per sample (12 root fragments). The number of sections where the AM formations arbuscules, vesicles, or hyphae were observed, was noted separately for each formation type. Dark or septate hyphae were not counted. The results were expressed as the proportion of root length colonised by arbuscules, vesicles, and hyphae, and proportion of uncolonised root, where no AM formations were encountered. Extent of AM colonisation by root fragment was separately analysed to obtain information on the proportion of completely empty or intensively colonised fragments. Colonisation was analysed for 10 cross sections per fragment and, consequently, the method yielded information on colonisation in 10 % classes.

Statistical analysis

Mycorrhizal colonisation was analysed as factorial design, with species and distance from tree rows as the factors. Colonisation results, expressed as percentage

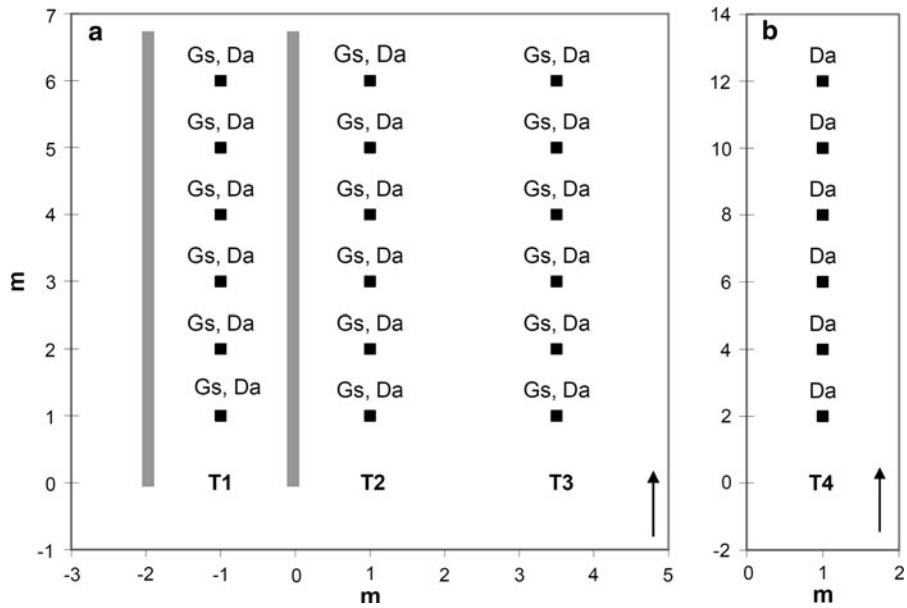

Fig. 1 Sampling scheme for mycorrhizal analyses on **a** the agroforestry plot. **b** the *Dichanthium aristatum* monocrop. The abbreviations T1–T4 refers to the sampling transects, and *Gs* and *Da* to the root samples of *Gliricidia sepium* and *D. aristatum*, respectively. *Grey bars* indicate the location of the *G. sepium* tree rows

(%), were normalised by arcsine transformation. The frequency distribution of the transformed data did not differ statistically significantly from the normal distribution for the studied variables, except for arbuscular colonisation and arbuscule:vesicle ratio in *G. sepium* (Kolmogorov–Smirnov test, $p < 0.05$). Variances did not differ statistically significantly between the species for any of the studied variables. Thus, AM colonisation between the two host plant species was compared with *t* test, and treatment means (sampling location) by using Tukey's test for least square means. Values of $p < 0.05$ were interpreted as indicating statistically significant differences. Results were analysed with SAS statistical analysis software, version 9.1 (SAS Institute Inc., Cary, NC, USA).

Identification of AM-forming fungi from root samples

DNA was extracted by CTAB and proteinase K buffer (Timonen et al. 1997) after vigorous pulverisation in liquid nitrogen with pestle and mortar. Nested PCR with outer primers SSUmAf: 5′-TTG GTA ATC TTD TGA AAC TTY-3′ and LSUmAr: 5′-GCT CTT ACT CAA AYC TAT CRA-3′ and inner primers SSUmCf: 5′-TAT TGY TCT TNA ACG AGG AAT G-3′ and LSUmBr: 5′-AA CAC TCG CAY AYA TGT TAG A-3′ were modified from Krüger et al. (2009). The PCR was carried out as in Krüger et al. (2009) with exception of annealing temperature 58 °C used in the nested PCR. PCR reactions were run into 1 % agarose-SYBR Green (Invitrogen) gel. The correct size products were cut cleanly from the gel under black light and frozen at −20 °C overnight. The liquid from the gel pieces was centrifuged into eppendorf tubes, precipitated with ethanol and sequenced at Macrogen Inc., Seoul, Republic of Korea. Acquired sequences were manually checked using Geneious Pro 4.5.5 (Biomatters Ltd., Auckland, New Zealand).

Reference sequences were retrieved from GenBank database and aligned with Geneious Pro 4.5.5. The phylogenetic analyses were performed by WinClada Ver. 1.00.08 (Nixon 2002) according to Timonen and Hurek (2006), with the following exceptions: maximum number of trees to keep [hold] 1 000 000, number of replications [mult*N] 5000, and starting trees per replications [hold] 20. A combined sequence of *Mortierella polycephala* sequences AB476414 and AF113464 was created to act as a fixed outgroup. The sequences retrieved from the examined roots were deposited to GenBank accession numbers FR873155, FR873164–FR873165 (*G. sepium*), and FR873156–FR873163 (*D. aristatum*).

Results

AM colonisation

Arbuscules were observed in all root samples, except for *G. sepium* roots from two locations. Average

arbuscular colonisation was 7.7 ± 0.9 % of root length for *G. sepium* and 13.1 ± 0.9 % for *D. aristatum*. No arbuscules were found in 62 % of all screened root fragments of *G. sepium* (10 per species and location), and in 47 % of root fragments of *D. aristatum* (Fig. 2). Arbuscules of *G. sepium* resembled the *Arum*-type, whereas in *D. aristatum* both *Arum*- and *Paris*-type arbuscules were observed (Fig. 3).

Arbuscular mycorrhizal colonisation and frequency of types of AM formations varied statistically significantly between species and sampling locations, but similarly with location for both species on the agroforestry plot, i.e. combined effects were not observed. *G. sepium* had significantly more vesicles and hyphae but less arbuscules than *D. aristatum* (Fig. 4a–c). Arbuscular colonisation of *G. sepium* was higher on the tree-grass plot (T1) than on the adjacent grass plot 3.5 m away from the trees (T3). Vesicle colonisation for both species and hyphal colonisation for *D. aristatum* were highest at 1 m from the tree rows (T2). Arbuscule:vesicle ratio decreased with distance from tree rows for both species, but it was always lower in *G. sepium* (Fig. 4d). Hyphal and total colonisation in *G. sepium* roots did not vary with distance from the tree rows. Arbuscule:vesicle ratio and arbuscular colonisation were lower in the *D. aristatum* monocrop (T4) than on the tree-grass plot (T1) or at 1 m from the tree rows (T2), respectively. Vesicle or hyphal colonisation did not differ between the two sites.

AM fungal species

Five different types of AM fungal sequences were found from the analysed root pieces (Fig. 5). Most of the sequences clustered into two different subgroups of *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler comb. nov. (ex *Glomus intraradices*; Schüßler and Walker 2010). One subgroup included sequences from both *G. sepium* and *D. aristatum*. All sequences from *D. aristatum* in this subgroup were from samples taken on the tree-grass plot (T1). The other subgroup included sequences from *D. aristatum* from both the agroforestry plot (T2) and the grass monocrop (T4). One *Paraglomus* sequence was detected from *D. aristatum* roots. Identity of two sequences retrieved from *G. sepium* remained unclear as they did not neatly cluster with any sequences available in the current databases.

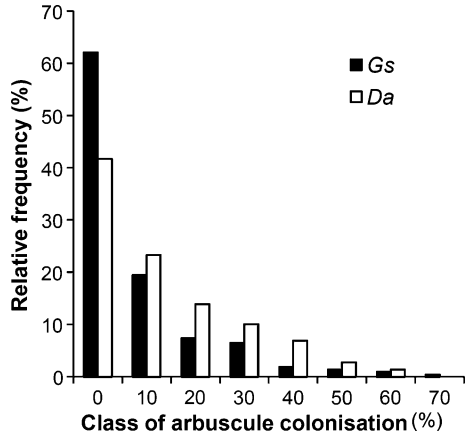

Fig. 2 Relative frequency of arbuscules by root fragment in *Gliricidia sepium* (*Gs*) and *Dichanthium aristatum* (*Da*). Arbuscule colonisation was determined from 10 cross sections in each root fragment and extent of colonisation by root fragment is therefore given is classes of 10 %

Discussion

AM colonisation along gradients of plant community composition and soil nutrient content

Our results add to the evidence that *G. sepium* forms symbiosis with AM-forming fungi in field conditions (Bakarr and Janos 1996; Okon et al. 1996; Kurppa et al. 2010). The results also confirm AM symbiosis in *D. aristatum*. Spores of AM-forming fungi have been previously analysed from soils cultivated with *D. aristatum* (Corcho et al. 2005). To our knowledge, mycorrhizal fungal species have not previously been identified with DNA sequencing techniques from field samples of *G. sepium* or *D. aristatum* roots.

Legumes rely less on mycorrhizal symbiosis for their N nutrition than non-legumes. However, they are often highly dependent on the fungal symbiont for acquisition of P required by dinitrogen fixation, and are also generally considered less efficient competitors for nutrients than grasses (Ledgard and Steele 1992), which may explain the overall differences in AM colonisation between the two plant species. Management practices can also affect colonisation: for example, frequent mowing of the host plant may restrict accumulation of carbon by the fungal symbiont, resulting in reduced vesicle formation (Titus and Leps 2000). This could partly explain the overall lower vesicle colonisation in *D. aristatum* roots in our

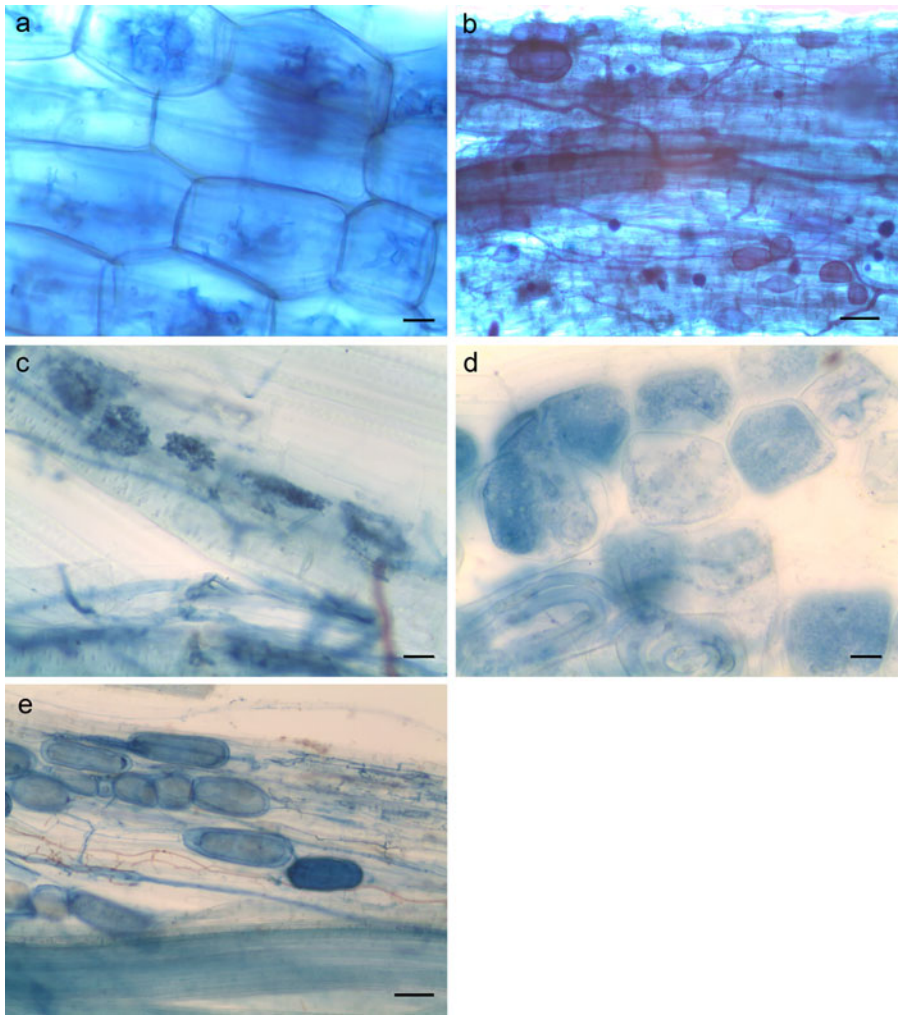

Fig. 3 Structures of arbuscular mycorrhizal fungi in root samples **a** *Arum-type* arbuscules and **b** vesicles in *Gliricidia sepium* roots, **c** *Arum*-type arbuscules, **d** arbusculate coils and *Paris*-type arbuscules, and **e** vesicles in *Dichanthium aristatum* roots. Bar: 10 μm (**a**, **c**, **d**) or 50 μm (a, **e**)

study, where the grass was frequently cut but several months had passed since the previous tree pruning.

Species of AM-forming fungi differ in their colonisation and spread patterns (e.g. Treseder and Allen 2002; Ingleby et al. 2007), and some groups of the fungi may associate more frequently with legumes than other plant species (Scheublin et al. 2007; Shepherd et al. 2007). Our results did not enable detailed analysis of differences in the AM fungal community with regard to host plant or sampling location, as sufficient fungal DNA could be extracted only from a few root samples. *R. intraradices* which was identified from both *G. sepium* and *D. aristatum* roots has been observed in a wide variety of ecosystems from tropical and temperate forests to grasslands, where it was commonly the dominant taxon, as well as in some arable lands, indicating that it is relatively tolerant to anthropogenic disturbance (Öpik et al. 2006). Observations of both *Arum*- and *Paris*-type arbuscules in *D. aristatum* roots suggest that the roots were colonised by more than one AM fungal species (reviewed in Dickson et al. 2007).

The fact that arbuscular mycorrhizal colonisation on the agroforestry plot varied similarly with distance from the tree rows for both *G. sepium* and *D. aristatum* could reflect a similar response among the plant species to soil nutrient content, especially P availability. Arbuscular colonisation for *G. sepium* was highest

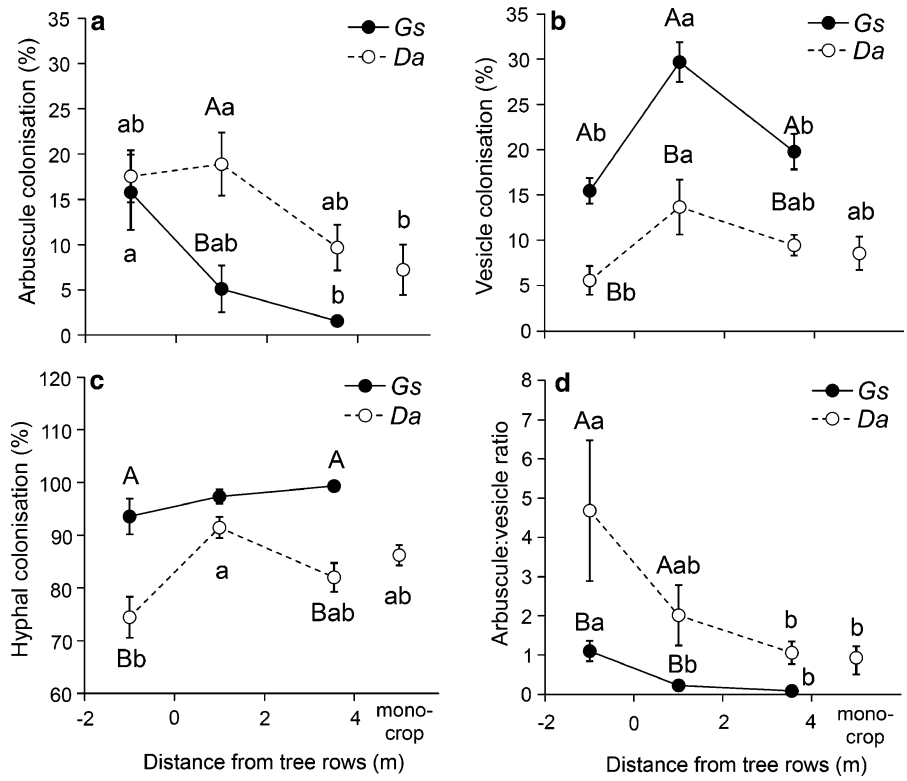

Fig. 4 Variation in arbuscular mycorrhizal colonisation by species and distance from nearest tree row (−1 = between tree rows): percentage of root length colonised by **a** arbuscules, **b** vesicles, **c** hyphae, and **d** arbuscule:vesicle ratio. *Gs* refers to *Gliricidia sepium* and *Da* to *Dichanthium aristatum*. Sampling points from left to right correspond to the sampling transects T1–T4. *Capital letters* indicate statistically significant differences between species at the given distance (*t* test), and *small letters* indicate within-species differences by distance (Tukey's test; $p < 0.05$ for both tests). *Vertical bars* indicate standard error ($n = 6$)

under the tree canopies where soil P content is lowest, and similar trend was observed for *D. aristatum*, although for it the differences were not statistically significant. Phosphorus limitation has been observed to increase arbuscular colonisation also in other ecosystems (Braunberger et al. 1991). Nitrogen availability may affect AM symbiosis on the plot less than P availability, as lowest AM colonisation in *D. aristatum* was observed away from the tree rows where soil N content also is at its lowest. Cost of maintaining the AM symbiosis to *D. aristatum* may overweigh the benefits where interspecific competition for nutrients is not intense, and therefore result in reduced colonisation.

Pattern of AM colonisation was probably affected not only by soil nutrient content, but also the relative densities of the two plant species and their competitive abilities, as implied by trends in arbuscule:vesicle ratio and vesicle colonisation. Mycorrhizal dependency of legumes may increase when they are intercropped with grasses, compared to a legume monocrop (Scheublin et al. 2007). The decreasing arbuscule:vesicle ratio in *G. sepium* with distance from the tree rows could indicate decreasing returns from the AM symbiosis under intensifying competition. Highest vesicle colonisation for both plant species was observed at 1 m from the tree rows (T2), the site where competition for nutrients between the species can be expected most even (Sierra and Nygren 2006; Daudin and Sierra 2008). Vesicle colonisation has been associated with increased carbon investments from the host plant to the fungal symbionts under nutrient-limiting conditions (Cooke et al. 1993); Treseder and Allen 2002). In *D. aristatum*, increased allocation of C to shoots under the shade of tree canopies could reduce C availability to the fungal symbiont, and explain the relatively low vesicle colonisation there. Shoot:root ratio of *D. aristatum* is known to be higher under the tree canopies than on the *D. aristatum* monocrop (Dulormne 2001).

Interestingly, in *D. aristatum* proximity to the legume trees appeared also to stimulate AM symbiosis, as arbuscular colonisation and the arbuscule:vesicle ratio in its roots were higher on the agroforestry plot than on the *D. aristatum* monocrop. The fact that on the agroforestry plot the plant species formed symbiosis with fungi belonging to the same *Rhizophagus intraradices* subgroup may also indicate stimulation of AM

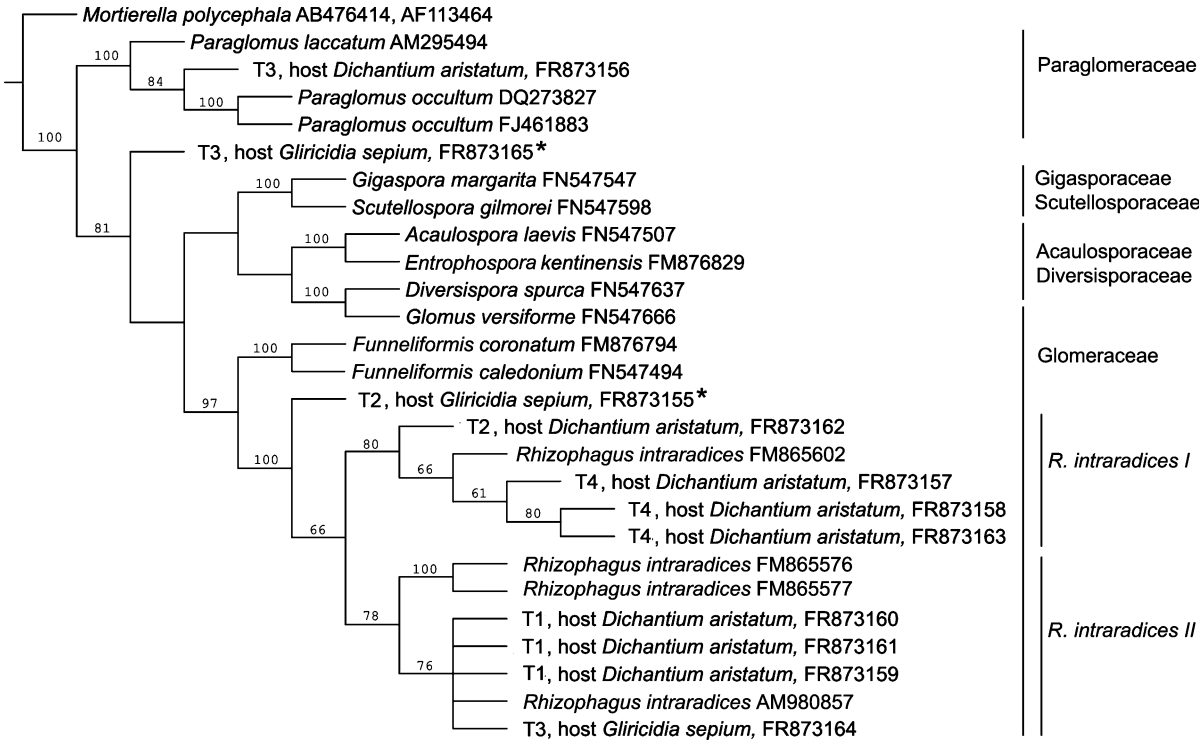

Fig. 5 Phylogenetic tree (partial small subunit, internal transcribed spacer, partial large subunit rDNA sequences) of arbuscular mycorrhizal fungi on the agroforestry field site, with *Mortierella polycephala* as outgroup. The strict consensus tree was created from five equally parsimonious trees (2978 steps, Ci 60, Ri 63). Jackknife values over 50, which indicate strong support for the branches, are marked above the branches. *Asterisks* (*) indicate the two samples from *G. sepium* whose identity remained unclear as they did not cluster with any sequences available from the current databases

symbiosis. Intercropping with a legume has been observed to stimulate AM colonisation also in the *Lolium perenne* grass (Barea et al. 1989).

The arbuscule:vesicle ratio has been suggested as an indicator of the relative cost or benefit of the fungus to the host plant. In previous studies it was found to be mostly affected by sampling time or plant age (Braunberger et al. 1991; Titus and Leps 2000). Our results indicate that the arbuscule:vesicle ratio may reflect differences in benefits of the AM symbiosis to the host plants also more directly, e.g. between plant species belonging to different functional groups or along gradients of nutrient availability and shading.

Environmental conditions and management practices can affect AM colonisation, both directly and through their effects on nutrient demand and availability (Titus and Leps 2000; Boddington and Dodd 2000). Biomass growth and nutrient uptake by *D. aristatum* are lowest during the dry season and after pruning of *G. sepium* (Daudin and Sierra 2008). Nitrogen mineralisation on the site varies as a function of temperature but not soil moisture (Sierra et al. 2002), and was probably relatively rapid during the time preceding sampling. Pruning induces changes in C and nutrient allocation within the tree, directing resources towards foliar regrowth (García et al. 2001) and reducing C supply to N_2-fixing nodules, which results in nodule turnover (Nygren and Cruz 1998). Symbiosis with AM-forming fungi may be similarly affected by pruning. Inhibition of N_2-fixation also increases demand for soil N by the tree and may prevent N transfer for several weeks (Nygren 1995; Daudin and Sierra, 2008). *G. sepium* was pruned last time 7 months and *D. aristatum* cut more than 40 days before sampling, which would imply relatively undisturbed AM symbiosis and efficient nutrient uptake by both species, and well-established dinitrogen fixation in *G. sepium* (Nygren et al. 2000). Competition for soil nutrients at the time of the sampling was, therefore, probably lower than during other times of the year or

tree management cycle, when more drastic differences in AM colonisation could be expected between species or sampling locations. Tree pruning in the studied cut-and-carry system is so frequent that flowering is impeded and the trees do not enter reproductive cycle (Nygren et al. 2000). Major temporal variation in AM colonisation patterns related to phenology is therefore not expected.

The low number of detected and identified AM fungi in root fragments could be explained by the difficulties in extraction of fungal DNA from the root samples as well as the low number of relatively small root fragments analysed. High fungal diversity in tropical soils may also confuse the use of non-specific primers (cf. Rosendahl 2008). The used primers should have fairly good coverage of the most common families of Glomales as shown by Krüger et al. (2009). Nevertheless, using specific primers for the individual families and species under Glomales could improve species identification in highly diverse fungal communities, when resources are available to cover the increased costs and labour requirements. This approach has been successfully applied for identifying AM fungal species from field samples in some tropical ecosystems (Wubet et al. 2003; Shepherd et al. 2007).

Implications to N transfer via common mycelial networks

Previous studies on the same fodder production site showed that N transfer from *G. sepium* to *D. aristatum* was highest next to the tree rows, despite of competition (Daudin and Sierra 2008). Symbiotically fixed N in *D. aristatum* shoots correlated statistically significantly with density of *G. sepium* roots, but not with soil isotopic signatures, indicating that N transfer was related to the presence of living *G. sepium* roots, and possibly to common mycelial networks of AM-forming fungi between the roots of the two species (Sierra and Nygren 2006). Nitrogen transfer via mycelial networks between the two plant species in a pot experiment correlated positively with arbuscular colonisation in *G. sepium* roots (Jalonen et al. 2009). The fact that both *G. sepium* and *D. aristatum* were observed to form symbiosis with the same subgroup of *R. intraradices* in our study implies favourable conditions for the formation of anastomoses between the fungal symbionts of the two plant species (Croll et al. 2009; Mikkelsen et al. 2008), although they have not yet been observed in the field. Thus, presence of the same AM fungal species in the roots of both plant species, and the high arbuscular colonisation in *G. sepium* roots next to the tree rows where highest N transfer has been observed suggest that at least part of the observed direct N transfer occurs via common mycelial networks of the AM-forming fungi.

Conclusions

We observed significant spatial variation in AM colonisation along a gradient of legume tree root density on the *G. sepium–D. aristatum* agroforesty plot. The results suggest that AM colonisation patterns on the plot are affected by soil nutrient content, but also by the competitive abilities of the two plant species and characteristics of the plant community at the sampling location. Intercropping appeared to stimulate AM colonisation in *D. aristatum*. Arbuscular colonisation for both species was highest under the tree canopies and near the tree rows, where most N transfer between the plant species has been observed in previous studies on the same plot. *R. intraradices* was observed in the roots of both species, indicating the possibility for anastomoses and N transfer via common mycelial networks of the AM fungal symbionts of the two plant species. Future studies should be designed to better understand how AM symbioses and their implications to mutualistic versus competitive relationships between intercropped plants are affected by soil nutrient content, seasonal variation and management practices such as tree pruning. Analysing patterns of arbuscular and vesicle colonisation separately, instead of recording only total AM colonisation, can contribute to understanding of functional variation in AM symbiosis. Finally, frequency of anastomoses between AM fungal symbionts of *G. sepium* and *D. aristatum* should be studied under controlled conditions, to complement the observations from field and greenhouse studies on the role of common mycelial networks in N transfer.

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