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Mycorrhizas in agroforestry: spread and sharing of arbuscular mycorrhizal fungi between trees and crops – complementary use of molecular and microscopic approaches.

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Key words: Calliandra calothyrsus, Gigaspora albida, Glomus etunicatum, molecular probes, tree-crop linkages

Abstract

The spread of arbuscular mycorrhizal (AM) fungi from tree to crop roots was examined by molecular and microscopic methods in a glasshouse study. Growth of *Calliandra calothyrsus* Meissner trees inoculated with isolates of the AM fungi *Glomus etunicatum* Becker & Gerdemann and *Gigaspora albida* Schenck & Smith was monitored over an 18 month period. Three successive 'intercrops' of beans or maize were sown at 25, 50 and 75 cm distances from the tree and harvested during this period. At each crop harvest, the distribution of tree and crop roots and the spread of the inoculant fungi were determined using traditional microscopic methods and fungal specific primers. Both inoculants greatly improved the growth of the trees and colonization spread to the crops once the trees were 6 months old. However, benefits of inoculation to crop growth were not observed due to increased competition from the larger inoculated trees growing in a restricted soil volume. Of the two inoculant fungi, *Glomus etunicatum* appeared to be more mobile as it spread more rapidly, formed higher levels of colonization at increasing distances from the tree and was responsible for most of the mycorrhizal cross-contamination. In contrast, colonization of tree and crop roots by *Gigaspora albida* was higher nearest the tree. This work demonstrated the benefits of mycorrhizal fungus inoculation for tree growth and confirmed that trees and crops share the same AM fungi. Trees may therefore act as reservoirs of mycorrhizal fungi, either inoculant or indigenous, for surrounding crops or other annual vegetation. It was also shown that tree pruning, the normal

practice in agroforestry systems, did not reduce mycorrhizal infection or prevent spread to crops. However, the slow rates of inoculant spread found here suggest that it may take years before inoculants benefit the growth of crops sown several metres from the tree. The work also demonstrated that microscopic quantification of mycorrhizal colonization and the use of molecular probes to identify specific fungi within roots can complement each other effectively. Molecular probes were more sensitive at detecting mycorrhizal fungi than microscopic methods, but did not discriminate between full mycorrhizal structures and traces of hyphae.

Abbreviations: AM – arbuscular mycorrhiza (l); RLD – root length density; PCR – polymerase chain reaction; BEG – International Bank for the Glomeromycota.

Introduction

Fast-growing, multipurpose tree species are widely planted on farms in semi-arid Africa as they perform a key role in stabilizing and improving farm soils while providing many additional and varied products such as timber, fodder and fruit, and increasing total farm productivity through exploitation of different niches, above and below ground (Sanchez et al., 1997). Many of the tree species employed are leguminous and form symbiotic associations with N_2-fixing bacteria (rhizobia) and arbuscular mycorrhizal (AM) fungi, which enable them to sustain growth in the phosphorus and nitrogen deficient soils typical of the region. These soils are often degraded through over-cultivation and erosion, and such intensification of land-use may lead to insufficient or ineffective populations of microsymbionts (Alvarez-Solis and Anzueto-Martinez, 2004). In these cases, inoculation with effective rhizobia and AM fungi may be needed for the re-establishment of trees, while long-term improvements in soil fertility and growth of the crops will require land management regimes which sustain and promote mycorrhizal populations (Sieverding, 1991).

As AM fungi are the predominant mycorrhizal type in dry tropical soils and associate with a wide range of plant species, they have the potential to benefit the growth of both tree and crop species in agroforestry systems. Tree legumes such as *Senna siamea*, *Gliricidia sepium* and *Calliandra calothyrsus* have shown high mycorrhizal dependency and respond to inoculation (Habte and Turk, 1991; Ingleby et al., 2001). Similarly, field crops such as cassava are known to be obligately dependent on AM fungi, and inoculation using several AM fungus inoculants has been highly beneficial to crop yields in a range of soils (Howeler et al., 1987). However, these responses vary widely according to the host species, the AM fungus inoculants used, soil fertility and the levels of indigenous populations of AM fungi, and these factors should be investigated before AM fungal inoculants are selected (Sieverding, 1991).

The importance of maintaining active populations of AM fungi in agroforestry soils in order to sustain crop productivity has also been demonstrated (Sieverding and Leihner, 1984; Dodd et al., 1990). More recently, Arihara and Karasawa (2000) have shown that maize yields were better and mycorrhizal fungus colonization higher in maize crops cultivated after other mycorrhizal crops, than in maize cultivated after non-mycorrhizal crops. AM fungus inoculum in the soil normally occurs as spores, mycorrhizal roots and mycelial networks, and Miller (2000) attributed early infection of maize seedlings and increased final grain yield to the key role AM mycelial networks play in enhancing phosphorus absorption in young plants. Although most sensitive to disturbance, AM mycelial networks are primarily responsible for the rapid colonization of new roots, and have been shown to retain their capacity to colonize roots even after long periods of drought typical of tropical regions (Brundrett and Abbott, 1994).

It is now widely accepted that AM mycelial networks form links between plant species in ecosystems, and that they are responsible for the transfer of nutrients between different plant species (Read, 1991). Haselwandter and Bowen (1996) proposed that AM fungi associated with agroforestry tree species may serve an additional role by maintaining active AM propagules in the soil, which could then rapidly colonize roots of emerging crop seedlings. Subsequent studies have supported this view: Leakey et al. (1999) reported that maize grown in soil taken from close to *Senna siamea* formed more mycorrhizas than when it was grown in soil collected at 2 m distance, while Diagne et al. (2001) examined soils from agroforestry systems in Senegal and found beneficial effects of *Acacia tortilis* trees on mycorrhizal fungus colonization and growth of millet seedlings. The role of perennial trees in maintaining AM fungus inoculum and in sustaining mycelial networks for short-lived crops may therefore be an unintended benefit of agroforestry systems and provide an alternative approach to the use of cover crops to build up soil inoculum.

This paper reports the results of a glasshouse study which examined the spread of AM fungi from tree to crop roots, and the resulting effects on plant growth. The experiment used *Calliandra calothyrsus*, a widely planted, multi-purpose, leguminous agroforestry tree species as the host tree, inoculated with two AM fungus inoculants and co-planted with maize or beans in sequence to simulate the cropping patterns in Kenya. 'Traditional' assessments of mycorrhizal colonization by staining and light microscopy were combined with molecular methods in order to accurately monitor the spread and distribution of the inoculant fungi.

Materials and methods

Design and set up of glasshouse experiment

On 6 February 2004, 75 cm^3 pots containing a sterilized loam/grit-sand mixture and 20 g of root/soil inoculum from either *Glomus etunicatum* Becker & Gerdemann (BEG 176) or *Gigaspora albida* Schenck & Smith (BEG 173) pot cultures, or an autoclaved mixture of these inoculants, were sown with *Calliandra calothyrsus* Meissner (Flores, ex. Maseno) seeds. These mycorrhizal fungus isolates originated from soil samples collected in proximity to *C. calothyrsus* in Honduras and Kenya respectively. Prior to registration with the International Bank for the Glomeromycota (BEG), they were known by their isolate numbers '*Glomus etunicatum* 1' and '*Gigaspora albida* 2' and had been shown to form mycorrhizas abundantly and promote the growth, shoot phosphorus and nodule dry mass of *C. calothyrsus* (Lesueur et al. 2001). After 11 days, germinating seedlings were thinned to one per pot and all seedlings were inoculated with 2 ml of a *Rhizobium* suspension comprising of two isolates also known to be effective with *C. calothyrsus* (isolates KWN35 & KCC6; Lesueur et al. 2001). Six weeks after inoculation, 3 seedlings were sampled from each treatment to examine their mycorrhizal status and the effectiveness of the inoculation procedure. Nine weeks after inoculation, the seedlings were transplanted, one per trough, to 100 x 20 x 20 cm troughs filled with a sterilized loam/grit-sand/coir mixture (3:3:1), pH 5.8, containing 63, 4.2 and 36 mg kg^{-1} of extractable NPK respectively, intended to simulate a P-deficient tropical soil. To improve drainage, the troughs were first lined with a 2-3 cm layer of coarse pebbles so that the actual depth of soil mixture in the troughs was approximately 15 cm. Seedlings were planted 7.5 cm from one end of the trough. The three inoculation treatments were replicated in eight randomised blocks, with each treatment represented once within each block. The troughs were located in a glasshouse set to provide a day/night temperature regime of 28/20^{o}C with high-pressure mercury vapour lamps to supplement natural sunlight and produce a day length of 14 h.

During the course of the study, crops were sown and harvested three times. On 2 June 2004 (15 weeks after AM fungus inoculation), *Phaseolus vulgaris* L. (seedlot Mwezi Moja GLP 1127 ex. Kenya 25/3/03) seeds were sown in the troughs 25 and 50 cm from the tree. After one week, emerging seedlings were thinned to one per distance. For this first cropping period, plants were harvested six weeks after sowing so that primary mycorrhizal colonization could be related to crop growth and the effects of the inoculation treatments. Subsequent cropping periods were extended to allow the crop plants to reach maturity before harvest, thus following cropping patterns in the field. On 6 September 2004 (28 weeks after AM fungus inoculation), *Zea mays* L. (seedlot H614D ex. Kenya 25/3/03) seeds were sown in the troughs 25 and 50 cm from the tree and thinned to one per distance as before. Plants were harvested 10 weeks after sowing. Finally, on 13 May 2005 (64 weeks after AM fungus inoculation), the trees were pruned to 30 cm height, removing most of the leaves and above-ground biomass of the inoculated plants, and the same seedlot of *Zea mays* was sown 25, 50 and 75 cm from the tree. Shoot pruning is regularly carried out in tropical agroforestry, and was done to evaluate its effects on mycorrhizal colonization and to reduce the intense tree-crop competition observed in the troughs in 2004. Plants were harvested 12 weeks after sowing.

Sampling and assessment

Growth of *C. calothyrsus* seedlings was monitored during the experiment by measuring stem diameter. Measurements were made every two weeks in 2004 and then every four weeks during 2005. Crop growth was assessed by taking weekly height measurements of the plants and measuring shoot dry weight at harvest. At harvest, crop shoots were severed at ground level, not uprooted. At the time of each crop harvest, two soil cores (1.6 cm diameter x 10 cm depth: approx. 20 cm^3 soil) were removed at each distance, and tree and crop roots were extracted for molecular and microscopic assessment of mycorrhizal fungus colonization. This coring depth focussed on the lateral and fine root development which was concentrated in the upper soil layers, with only coarse tap roots developing through the pebbles at the base of the troughs. The root distribution in the troughs was confirmed after 28 weeks with the destructive harvest of troughs from block two, in which the uninoculated tree had become contaminated by *Glomus etunicatum*. In 2004, cores were removed at 0, 25 and 50 cm from the tree, and at 0, 25, 50 and 75 cm in 2005. Four, seven and six blocks were assessed in July 2004, November 2004 and August 2005 respectively. Coring holes were re-filled with the same soil mixture and care was taken to avoid re-filled holes on subsequent sampling occasions. Root sampling for molecular work demanded a rigorous approach in order to ensure that hyphal fragments did not cross-contaminate the samples: corers and all other implements used were surface sterilised between each sample. Soil from the two cores was bulked for each distance and spread in sterile 14 cm Petri dishes. Roots were first removed aseptically, washed in sterile water and separated into tree and crop fractions. These fractions were then cut into 1 cm root fragments and mixed, before 10 fragments were randomly sampled and transferred to Eppendorf tubes for DNA extraction. The remaining roots were stained in Trypan blue (Koske and Gemma, 1989) prior to assessment of root length and the proportion that was mycorrhizal, using the gridline intersect method (Tennant, 1975). Root samples were used preferentially for molecular analysis and, in a few instances, insufficient roots remained for assessment of mycorrhizal colonization. As mycorrhizal colonization in *C. calothyrsus* roots was often difficult to observe under the dissecting microscope, sub-samples of these roots were mounted on glass slides to confirm the presence of colonization under the compound microscope. Root length density (RLD) (cm root 100 cm^{-3} soil) was calculated.

Data analysis

For tree growth, a one-way analysis of variance (ANOVA) was used, with inoculation as the treatment factor. For all other parameters, differences between treatments were examined by 2-way ANOVA using inoculation and distance from the tree as treatment factors. Data were examined for normality (Anderson-Darling, Cramer-von Mises and Watson tests; Stephens, 1974), homogeneity of variances (Bartlett's test; Sokal and Rohlf, 1995), and transformed where necessary to conform with the requirements of ANOVA. Differences between means were compared using Fisher's LSD test when the F-test from ANOVA was significant at $P \leq 0.05$.

Use of molecular probes

DNA was extracted from the roots using a Qiagen DNeasy plant mini kit after grinding for 30 s at 30 Hz in a Retsch MM300 grinder. DNA extracts were quantified by eye after electrophoresis in 1% agarose gel and either retained as neat extracts or diluted 1:20 with deionised water. Extracts were used as template DNA for amplification by polymerase chain reaction (PCR): for each sample, PCR was carried out in triplicate and, at each stage, water samples and DNA extracts from spores of the two inoculant fungi were included as negative and positive controls respectively. To test for the presence of the inoculant fungi, nested PCRs were performed using the universal primers ITS1 (White et al., 1990) and NDL22 (van Tuinen et al., 1998) at the first stage, and primers developed for *Glomus etunicatum* BEG 176 and *Gigaspora albida* BEG 173 (Walters and MacDonald, unpublished) at the second stage. Template DNA for the second stage PCR consisted of 2.0 µl of pure PCR product from stage 1. At both first and second stage, 25 µl PCR reactions contained 2.0 µl template DNA, 2.5 µl of 10 mM dNTPs (Promega), 1.0 µl of each 25 µM primer (MWG Biotech), 0.5 μl of 0.4 μg μl^{-1} bovine serum albumin, 2.5 μl 10X PCR buffer (New England Biolabs), 1U Taq DNA polymerase (New England Biolabs) and 15.3 μl deionised water. Reactions were covered with foil seals and run on a ThermoHybaid MBS 0.2G Thermal Cycler for 1 denaturing step of 94 °C for 5 mins then 30 cycles of 94 °C for 60 secs, 58 °C for 60 secs, 72 °C for 60 secs and a final extension step of 72 °C for 10 min. PCR products were visualised by electrophoresis on 1% agarose gels. A successful amplification in any one of the three triplicate PCRs was considered to indicate presence of the target fungus: we considered triplication as representing sampling power rather than PCR verification, which was provided by successful positive control amplification.

Results

Tree growth

From the time of transplanting to the troughs in 2004 until the end of the experiment in 2005, *C. calothyrsus* seedlings inoculated with *G. etunicatum* and *G. albida* were significantly (P<0.001) greater in stem diameter than the uninoculated control tree seedlings (Figure 1). No significant differences were observed between trees inoculated with *G. albida* and those inoculated with *G. etunicatum*. Figure 1 also indicates a reduction in the growth rate of the inoculated trees after about 40 weeks.

Crop growth

In July 2004, shoot dry weight of *P. vulgaris* harvested after six weeks was not significantly affected by inoculation treatment or distance from the tree (Table 1). In November 2004, shoot dry weight of *Z. mays* plants after 10 weeks was significantly (P<0.001) higher at 50 cm distance from the tree than at 25 cm, indicating that crops growing closest to the trees were suffering from competition, especially with the larger inoculated trees. In August 2005, shoot dry weight of *Z. mays* after 12 weeks was significantly (P<0.001) higher in the uninoculated troughs where trees were smaller.

However, growth was much better across all treatments, suggesting that shoot pruning of the trees in May 2005 had reduced competition, especially from the larger inoculated trees.

Root growth

In July 2004, tree RLD was greatest on inoculated trees (P<0.001) and nearest the tree (P<0.001), whereas crop (*P. vulgaris*) RLD was greatest further away from the tree (P<0.001) (Table 2). Similar differences were observed in November 2004 and August 2005, when *Z. mays* plants were harvested. However, after tree pruning in 2005, concentrations of crop roots found near the tree were much higher than those found in 2004. The results also show that, by 2005, roots of inoculated trees had extended throughout the trough.

Mycorrhizal colonization

Six weeks after inoculation, and prior to transplanting into the troughs, both inoculants had formed mycorrhizas on the *C. calothyrsus* seedlings: those inoculated with *G. etunicatum* had 12% of their root length colonized, while those inoculated with *G. albida* had 40%.

Subsequently, in July 2004, mycorrhizal colonization of tree roots was greatest nearest the tree *(P<0.001)*, but was not found in any crop roots, although very few crop roots were found near the tree where most tree root mycorrhizal colonization occurred (Table 2). Although significant differences between inoculation treatments were absent *(P = 0.057)*, colonization of *G. albida* inoculated trees close to the stem remained at 40%, while that of *G. etunicatum* inoculated trees was 31%. In November 2004, a significant inoculation x distance interaction was found for mycorrhizal colonization of tree roots with colonization of *G. albida* inoculated trees greater than that of *G. etunicatum* inoculated trees at 0 and 25 cm from the tree. Although both inoculants had colonized roots at 50 cm from the tree, colonization was greatest nearest the tree and decreased at 25 and 50 cm from the tree. By this time, mycorrhizal colonization was present on crop roots at 25 cm from the tree in both the inoculation treatments.

In August 2005, mycorrhizal colonization of tree roots followed a similar pattern to the previous November. Mycorrhizal colonization of crop roots was also greatest in inoculated troughs and nearest the tree. However, a significant inoculation x distance interaction (P<0.001) showed that although levels of colonization of crop roots by *G. albida* remained higher than those of *G. etunicatum*, and those growing with uninoculated trees remained the lowest, colonization of *G. albida* crop roots decreased at 50 and 75 cm from the tree whereas colonization by *G. etunicatum* was more consistent and only decreased at 75 cm from the tree. The results in August 2005 also showed that high levels of colonization were present on both tree and crop roots despite the heavy pruning of the trees prior to sowing this crop. Although some mycorrhizal colonization was found in tree and crop roots from uninoculated troughs, the more detailed data presented in Figures 2 - 4 shows that this was sporadic colonization of individual plants rather than widespread contamination.

Rate of spread and molecular identification of the inoculant fungi

In order to compare the results from microscopic and molecular assessments, this section presents data from the individual troughs rather than treatment means. Figures 2-4 show the % of colonization as determined by conventional staining, and the identity of the causal AM fungi as determined by the molecular probes. These assessments were made on parallel sub-samples of roots, so that the figures indicate the level of mycorrhizal fungus colonization in each sample and the presence or absence of the two inoculant fungi.

At the time of the *P. vulgaris* crop harvest, 21 weeks after inoculation of the trees and 6 weeks after crop sowing, levels of mycorrhizal fungus colonization in *C. calothyrsus* roots sampled at 0 cm varied from 21-51% for those inoculated with *G. etunicatum* and from 20-52% for those inoculated with *G. albida* (Fig. 2a-c). Although roots of the large, inoculated *C. calothyrsus* seedlings had extended beyond 50 cm (Table 2), only sporadic colonization was detected beyond 0 cm, and colonization of crop roots was negligible. The molecular probes indicated that *G. albida* had not yet extended to 25 cm from the tree, whereas *G. etunicatum* was present in one trough at 50 cm. The molecular probes also indicated that the mycorrhizal fungus colonization observed at 0 cm on the uninoculated *C. calothyrsus* seedling in block two was attributable to *G. etunicatum* (Fig. 2c).

Assessments of tree and crop root samples from the harvest of the second crop in November 2004 showed that both inoculant fungi had colonized tree roots at 25 cm and had spread to the crop roots (Fig. 3a,b). Tree roots had now extended more than 75 cm from the tree, but neither inoculant fungus had established a significant presence on the tree roots at 50 cm, although *G. etunicatum* was present in three of the root samples. Mycorrhizal fungus colonization was recorded in two uninoculated troughs (Fig. 3c), but the fungal specific primers did not detect either of the inoculant fungi on the roots, indicating that other AM fungi present in the glasshouse may have been responsible.

By August 2005, both inoculant fungi had colonized tree roots at 50 cm and to a lesser extent at 75 cm and, when present, had successfully spread to the crop roots at these distances (Fig. 4a,b). Crops had higher mycorrhizal colonization at 75 cm from the trees with *G. etunicatum* inoculation than with *G. albida*, and the spread of *G. etunicatum* from the tree to the crop appears to have been more consistent than that of *G. albida* (Table 2, Fig. 4a,b), even though differences in tree RLD were not found between the two inoculation treatments at these distances. As it was more than 16 months since the inoculated *C. calothyrsus* seedlings were transplanted to the troughs, it was perhaps not surprising that mycorrhizal cross-contamination had occurred in several troughs by this time. Of the two inoculant fungi, most cross-contamination was attributable to *G. etunicatum*. This inoculant was responsible for the contamination of two troughs inoculated with *G. albida*, whereas only one trough inoculated with *G. etunicatum* was contaminated by *G. albida*. At this time, three uninoculated troughs were contaminated by either *G. etunicatum* or *G. albida*.

Rates of spread were calculated for the inoculant fungi from the starting position of the transplanted tree seedling to their positions as detected by molecular probes at different times during the study. For 2004, rates for *G. etunicatum* were 1.2-2.5 mm d^{-1} and for *G. albida* were 1.2 mm d^{-1}. Over the whole experiment, rates of spread were between 1.1 and 1.6 mm d^{-1}.

Over the course of the experiment, the molecular probes consistently differentiated between the two inoculant fungi and appeared to be more sensitive than microscopic assessment in the detection of the inoculant fungi in the roots. In the inoculated troughs, the molecular probes detected the inoculant fungi in 17 root samples in which no mycorrhizal fungus colonization was observed under the microscope. In comparison, mycorrhizal colonization was only observed in 12 samples in which the molecular probes failed to detect the inoculant fungi. Given that the PCRs were performed in triplicate, and that DNA extracted from spores of the inoculant fungi was used as positive controls, it is most likely that mycorrhizal colonization in the absence of molecular detection was attributable to other AM fungi present in the glasshouse.

Discussion

As previously reported (Lesueur et al., 2001), *Calliandra calothyrsus* responded well to mycorrhizal fungus inoculation using these AM fungal isolates, and growth was poor in the controls despite rhizobial inoculation. Although *Gigaspora albida* formed more mycorrhizas on *C. calothyrsus* than *Glomus etunicatum*, both were similarly effective in promoting tree growth. As roots of the larger inoculated trees had already extended almost the length of the troughs after 30 weeks, the reduction in growth rate of the inoculated trees after 40 weeks is attributed to the trees becoming increasingly pot-bound.

Although mycorrhizal fungus inoculation had clearly stimulated growth of the trees, strong tree-crop competition restricted growth of the crops in the restricted soil volume of the troughs. By 2005, the inoculated tree roots had extended throughout the trough and would have been in direct competition with crop roots for nutrients and water at all sampling locations. The increase in crop RLD and shoot growth near the trees in 2005 compared to those in 2004, suggests that tree shoot pruning successfully reduced competition. However, the high levels of colonization found on both tree and crop roots indicate that shoot pruning had not impaired the viability of the AM inoculants. This is encouraging, as it suggests that normal tree management procedures will not damage the activity of AM fungus inoculum in agroforestry systems. Although work by Whitcomb and Stutz (2001) suggests that shoot pruning reduces tree root biomass and levels of AM colonization, it is not known whether shoot pruning, and the concomitant reduction of C supplied to roots, would slow the spread of AM fungi in the soil. It is more likely that tree root pruning, also used to control below-ground competition, and tillage, which destroys most tree roots in the top 10-15 cm of soil (Rao et al., 2004), will have adverse effects on the spread and transfer of AM fungi to crop plants. Both of these practices are subjects requiring longer-term studies in field plots.

The spread of both inoculant fungi on the tree roots was slower than expected, given that mycorrhizas were established on the tree roots at the time of transplanting to the troughs and could provide an immediate base from which AM hyphae could spread through the soil. Although this study did not involve *in situ* observations of fungal mycelia, rates of spread were determined by mycorrhizal colonization and presence of the inoculant fungi on the roots. These rates of spread (1-2.5 mm d^{-1}) are low compared to the observations of Jakobsen (1992) and Jansa et al. (2005) (determined by direct hyphal observation and indirectly through measurements of P acquisition, respectively) where hyphal growth rates of 1.5 – 3.2 mm d^{-1} through soil from which roots were excluded were measured. In this trough experiment, we would expect faster rates of spread as roots were not excluded and colonized roots would have assisted the spread of the fungi. These rates of spread may overestimate that which would occur in the field, as factors such as seasonal stresses, competition from other AM fungi and soil microbes, lower root length densities (Odhiambo et al., 1999; Olsson and Wilhelmsson, 2000) and disruption of mycelial networks through hand or machine tillage (Kabir, 2005), might slow the spread of inoculants. On the other hand, random dispersal of AM propagules through wind, water, animal or human activity was strictly controlled in the glasshouse. Nevertheless, these results suggest that it may take years before AM fungus inoculants benefit the growth of crops sown several metres from the tree.

Of the two inoculant fungi, *G. etunicatum* appeared to be the more mobile as it spread more rapidly through the troughs, established higher levels of colonization on the crops at increasing distance from the tree, and was responsible for more cross-contamination of troughs. In contrast, *G. albida* formed higher levels of colonization on tree and crop roots nearest the tree. These observations support the work of Voets et al., (2006) who reported the contrasting behaviour of developing mycelial networks in Glomeraceae and Gigasporaceae and their divergent strategies for the exploration and exploitation of new substrates.

This work has also demonstrated that microscopic quantification of colonization and the use of molecular probes to identify specific AM fungi within roots can complement each other effectively. The fungal specific primers we used as molecular probes consistently differentiated between the 2 inoculant fungi, and showed greater sensitivity in detection of the inoculant fungi in root samples compared with the traditional microscopic methods of assessment. Molecular methods were therefore more sensitive, detecting fungal fragments and enabling positive identification of the fungal isolates, whereas microscopy allowed discrimination between functioning and non-functioning mycelia on the basis of mycorrhizal structures observed within the root. However, although the molecular primers were developed to be “isolate-specific”, it is possible that they may have amplified sequences from other isolates of the same species or even other species. As sequence length (number of base pairs) should differ between species, we would anticipate successful detection of non-specific amplification but, in the case of conspecific isolates, homologous fragments may be produced, particularly from field samples. We therefore recommend that fragment specificity should be confirmed by sequencing and that further primer development is undertaken to verify and improve the degree of isolate specificity.

This study has shown that trees can potentially act as reservoirs of either inoculated or indigenous

AM inoculum, even though rates of spread of the inoculant fungi were slow. The experiment demonstrated the difficulty in promoting mycorrhizal activity on tree roots in order to obtain early mycorrhizal formation on crop plants, while avoiding competition for water and nutrients between tree and crop roots. Competition also occurs under field conditions where soil volumes are not restricted, and further work is needed to develop land management methods which reduce tree-crop competition and promote the activity of mycorrhizal propagules in the soil.

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References

Alvarez-Solis J D and Anzueto-Martinez M D 2004 Soil microbial activity under different corn cropping systems in the highlands of Chiapas, Mexico. Agrociencia 38:13-22.

Arihara J and Karasawa T 2000 Effect of previous crops on arbuscular mycorrhizal formation and growth of succeeding maize. Soil Sci. Plant Nutr. 46: 43-51.

Brundrett M C and Abbott L K 1994 Mycorrhizal fungus propagules in the jarrah forest. New Phytol. 127: 539-546.

Diagne O, Ingleby K, Deans J D, Lindley D K, Diaite I and Neyra M 2001 Mycorrhizal inoculum potential of soils from alley cropping plots in Senegal. Forest Ecol. Manage: 146, 35-43.

Dodd J C, Arias I, Koomen I and Hayman D S 1990 The management of populations of vesicular-mycorrhizal fungi in acid-infertile soils of a savanna ecosystem. I. the effect of pre-cropping and inoculation with VAM-fungi on plant growth and nutrition in the field. Plant Soil 122: 229-240.

Habte M and Turk D 1991 Response of two species of *Cassia* and *Gliricidia sepium* to vesicular-arbuscular mycorrhizal infection. Comm. Soil Sci. Plant Anal. 22: 17-18.

Haselwandter K and Bowen G D 1996 Mycorrhizal relations in trees for agroforestry and land rehabilitation. For. Ecol. Manage: 81, 1-17.

Howeler R H, Sieverding E and Saif S 1987 Practical aspects of mycorrhizal technology in some tropical crops and pastures. Plant Soil 100: 249-283.

Ingleby K, Fahmer A, Wilson J, Newton A C, Mason P A and Smith R I 2001 Interactions between mycorrhizal colonisation, nodulation and growth of *Calliandra calothyrsus* seedlings supplied with different concentrations of phosphorus solution. Symbiosis 30: 15-28.

Jakobsen I, Abbott L K and Robson A D 1992 External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 1. Spread of hyphae and phosphorus inflow into roots. New Phytol. 120: 371-380.

Jansa J, Mozafar A and Frossard E 2005 Phosphorus acquisition strategies with arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276: 163-176.

Kabir Z 2005 Tillage or no-tillage: impact on mycorrhizae. Can. J. Plant Sci. 85:23 – 29.

Koske R E and Gemma J N 1989 A modified procedure for staining roots to detect VA mycorrhizas. Mycol. Res. 92: 486-505.

Leakey R R B, Wilson J and Deans J D 1999 Domestication of trees for agroforestry in drylands. An. Arid Zone 38: 195-220.

Lesueur D, Ingleby K, Odee D, Chamberlain J, Wilson J, Manga T T, Sarrailh J M and Pottinger A 2001 Improvement of forage production in *Calliandra calothyrsus*: methodology for the identification of an effective inoculum containing Rhizobium strains and arbuscular mycorrhizal isolates. J. Biotech. 91: 269-282.

Miller M H 2000 Arbuscular mycorrhizae and the phosphorus nutrition of maize: A review of Guelph studies. Can. J. Plant Sci. 80: 47-52.

Odhiambo H O, Ong C K, Wilson J, Deans J D, Broadhead J and Black C 1999 Tree-crop interactions for below-ground resources in drylands: root structure and function. An. Arid Zones 38: 221-237.

Olsson P A and Wilhelmsson P 2000 The growth of external AM fungal mycelium in sand dunes and in experimental systems. Plant Soil 226: 161-169.

Rao M K, Schroth G, Williams S E, Namirembe S, Schaller M and Wilson J 2004 Managing below-ground interactions in agroecosystems. In: van Noordwijk M, Cadisch G and Ong C K (eds) Below-ground interactions in tropical agroecosystems. Concepts and models with multiple plant components. CABI Publishing, Wallingford, pp 309 – 328

Read D J 1991 Mycorrhizas in ecosystems. Experientia 47: 376-391.

Sanchez P A, Buresh R J and Leakey R R B 1997 Trees, soils, and food security. In Phil. Trans. Royal Soc. B: Biol. Sci. 352: 949 – 961.

Sieverding E and Leihner D E 1984 Influence of crop rotation and intercropping of cassava with legumes on VA mycorrhizal symbiosis of cassava. Plant Soil 80: 143-146.

Sieverding E 1991 Vesicular-arbuscular mycorrhiza management in tropical ecosystems. GTZ, Eschborn, Germany. pp. 371.

Sokal R R and Rohlf F J 1995 Biometry: the principles and practice of statistics in biological research. W.H. Freeman, San Francisco. pp. 887.

Stephens M A 1974 EDF statistics for goodness of fit and some comparisons. J. Am. Stat. Assoc. 69: 730-737.

Tennant D 1975 A test of a modified line intersect method of estimating root length. J. Ecol. 63: 995-1001.

van Tuinen D, Jacquot E, Zhao B, Gollotte A and Gianinazzi-Pearson V 1998 Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7: 879-887.

Voets L, de la Providencia IE and Declerck S 2006 Glomeraceae and Gigasporaceae differ in their ability to form hyphal networks. New Phytol. 172: 185-188.

Whitcomb S A and Stutz J 2001 Effects of pruning on root length density, root biomass, and arbuscular mycorrhizal colonization in two landscape shrubs. Proc. 3rd International Conference on Mycorrhizas, Abs: 1-157.

White T J, Bruns T, Lee S and Taylor J 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols, a guide to methods and applications. Eds. Innis M A, Gelfand D H, Sminski J J and White T J. pp. 315-322. Academic Press, San Diego.

Table 1. Shoot dry weight (g) of crop plants grown at different distances from *Calliandra calothyrsus* trees inoculated with 2 different AM fungal isolates or left uninoculated. Values are means of 4 (July 04), 7 (Nov 04) and 6 (Aug 05) replicates.

Inoculation treatment	*Glomus etunicatum*			*Gigaspora albida*			Uninoculated			*P* value .		
Distance from tree (cm)	25	50	75	25	50	75	25	50	75	Inoc.	Dist.	Inoc. x Dist.
P. vulgaris harvest July 2004	2.60	2.46	n.a.[1]	2.58	2.72	n.a.	2.66	3.33	n.a.	0.403	0.445	0.513
Z. mays harvest November 2004	0.99	3.75	n.a.	0.75	3.92	n.a.	1.37	4.05	n.a.	0.833	<0.001	0.928
Z. mays harvest August 2005	7.5	5.0	16.6	3.7	6.0	5.9	11.2	18.2	17.4	<0.001	0.075	0.229

[1] samples were not assessed (n.a.) at 75 cm distance in July and November 2004.

Table 2. Root length density (cm 100 cm^{-3} soil) and mycorrhizal infection (% root length) of tree and crops at the time of three crop harvests made during 2004 - 2005.

Values are means of 4 (July 04), 7 (Nov 04) and 6 (Aug 05) replicates.

Inoculation treatment		*Glomus etunicatum*				*Gigaspora albida*				Uninoculated				*P* value[1]		
Distance from tree (cm)		0	25	50	75	0	25	50	75	0	25	50	75	Inoc.	Dist.	Inoc. x Dist.
Root length density	July 04	188	86	76	n.a.[2]	301	73	40	n.a.	69	4	0	n.a.	<0.001	<0.001	0.500
Tree	Nov 04	362	156	147	n.a.	841	261	81	n.a.	102	4	0	n.a.	<0.001	<0.001	0.113
	Aug 05	582	481	180	104	767	417	115	87	191	64	34	0	<0.001	<0.001	0.331
Root length density	July 04	3	68	229	n.a.	4	63	186	n.a.	33	75	231	n.a.	0.142	<0.001	0.710
Crop	Nov 04	5.2d	105b	141ab	n.a.	6.5d	61.2c	151a	n.a.	23.4d[3]	136ab	125ab	n.a.	0.128	<0.001	0.007
	Aug 05	208	372	412	415	242	214	323	427	453	573	676	582	<0.001	0.016	0.703
Mycorrhizal colonization	July 04	31.0	8.3	2.6	n.a.	40.6	4.7	0.4	n.a.	17.3	3.6	0	n.a.	0.057	<0.001	0.549
Tree	Nov 04	28.4b	6.1c	0.9d	n.a.	48.6a	24.8b	1.1d	n.a.	2.5cd	0d	0d	n.a.	<0.001	<0.001	<0.001
	Aug 05	29.1	23.3	6.1	2.5	42.6	40.7	10.5	0.1	7.1	3.1	0	0	<0.001	<0.001	0.159
Mycorrhizal colonization	July 04	n.r.	0	0	n.a.	n.r.[4]	0	0	n.a.	0	0	0	n.a.	-	-	-
Crop	Nov 04	n.r.	20.5a	1.6b	n.a.	n.r.	19.3a	0.4b	n.a.	11.0b	0b	0.6b	n.a.	0.199	<0.001	<0.001
	Aug 05	45.6b	48.8b	44.7b	27.9c	75.0a	71.9a	35.8bc	10.7d	4.4de	6.3de	0e	0e	<0.001	<0.001	<0.001

[1] square root and angular transformations were performed on root length density and mycorrhizal colonization for statistical analysis; untransformed means are shown in this table.

[2] root samples were not assessed (n.a.) at 75 cm distance in July and November 2004.

[3] letters indicate significant differences within each row for the inoculation x distance interaction as determined by Fisher's LSD test, when $P<0.05$ as determined by ANOVA.

[4] no roots (n.r.) were present in these samples.

Figure 1. Stem diameter of inoculated and uninoculated *Calliandra calothyrsus* trees in a glasshouse trough experiment during 2004-2005 (error bars = ±SE (n=7); horizontal bars indicate cropping periods)

Figure 2 a-c. Extent of mycorrhizal colonization (% root length) determined microscopically, and origin (*G. etunicatum*, *G. albida* or other) of mycorrhizal fungus determined by molecular methods, on roots of trees *(C. calothyrsus)* and crops *(P. vulgaris)* growing together in troughs. Samples collected in July 2004 from cropping period C1. Trees were previously inoculated with (a) *G. etunicatum*, (b) *G. albida* or (c) not inoculated. Samples were taken at different distances from the tree (0, 25 and 50 cm) in 4 replicate troughs. X axis shows block numbers of samples taken at different distances. Data for trees and crops taken from the same soil cores are presented in adjacent columns. The presence of a small coded section at the top of a bar indicates molecular confirmation of one of the two inoculant fungi.

Figure 3 a-c. Extent of mycorrhizal infection (% root length) determined microscopically, and origin (*G. etunicatum*, *G. albida* or other) of mycorrhizal fungus determined by molecular methods, on roots of trees *(C. calothyrsus)* and crops *(Z. mays)* growing together in troughs. Samples collected in November 2004 from cropping period C2. Trees were previously inoculated with (a) *G. etunicatum*, (b) *G. albida* or (c) not inoculated. Samples were taken at different distances from the tree (0, 25 and 50 cm) in 7 replicate troughs. X axis shows block numbers of samples taken at different distances. Data for trees and crops taken from the same soil cores are presented in adjacent columns. The presence of a small coded section at the top of a bar indicates molecular confirmation of one of the two inoculant fungi.

Figure 4 a-c. Extent of mycorrhizal infection (% root length) determined microscopically, and origin (*G. etunicatum*, *G. albida* or other) of mycorrhizal fungus determined by molecular methods, on roots of trees *(C. calothyrsus)* and crops *(Z. mays)* growing together in troughs. Samples collected in August 2005 from cropping period C3. Trees were previously inoculated with (a) *G. etunicatum*, (b) *G. albida* or (c) not inoculated. Samples were taken at different distances from the tree (0, 25, 50 and 75 cm) in 6 replicate troughs. X axis shows block numbers of samples taken at different distances. Data for trees and crops taken from the same soil cores are presented in adjacent columns. The presence of a small coded section at the top of a bar indicates molecular confirmation of one of the two inoculant fungi.

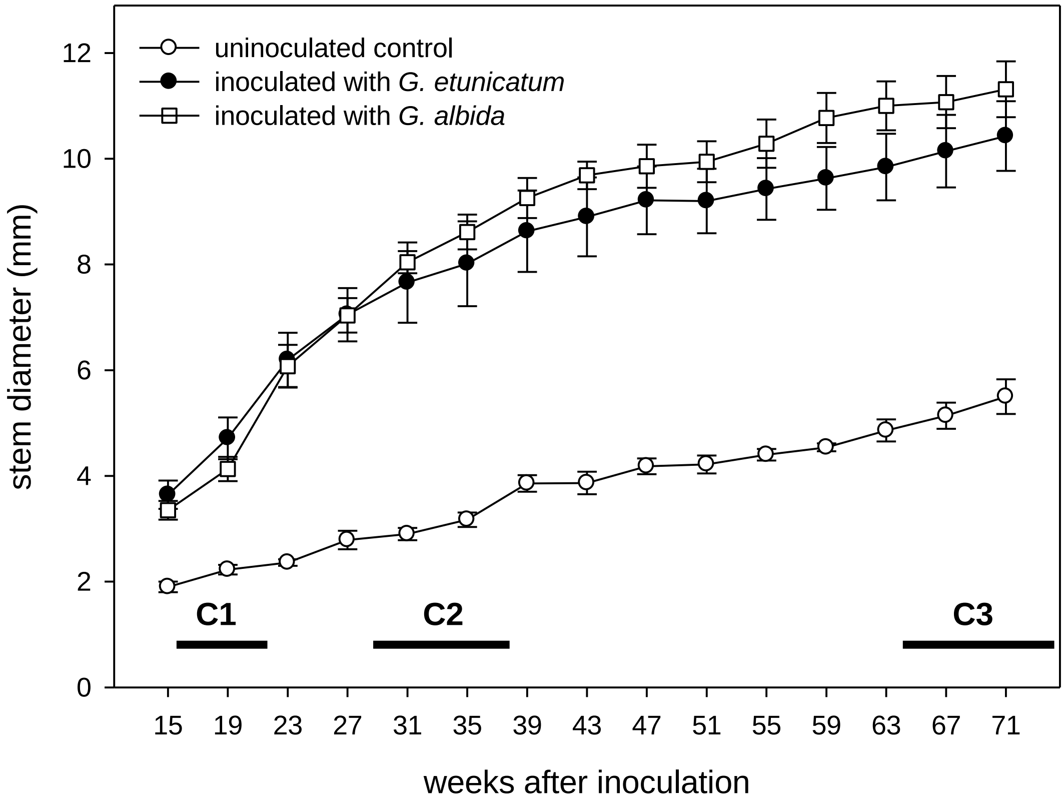
uninoculated control
inoculated with *G. etunicatum*
inoculated with *G. albida*
stem diameter (mm)
12
10
8
6
4
2
0
C1
C2
C3
15
19
23
27
31
35
39
43
47
51
55
59
63
67
71
weeks after inoculation

Figure 2a. Trees inoculated with *G. etunicatum*

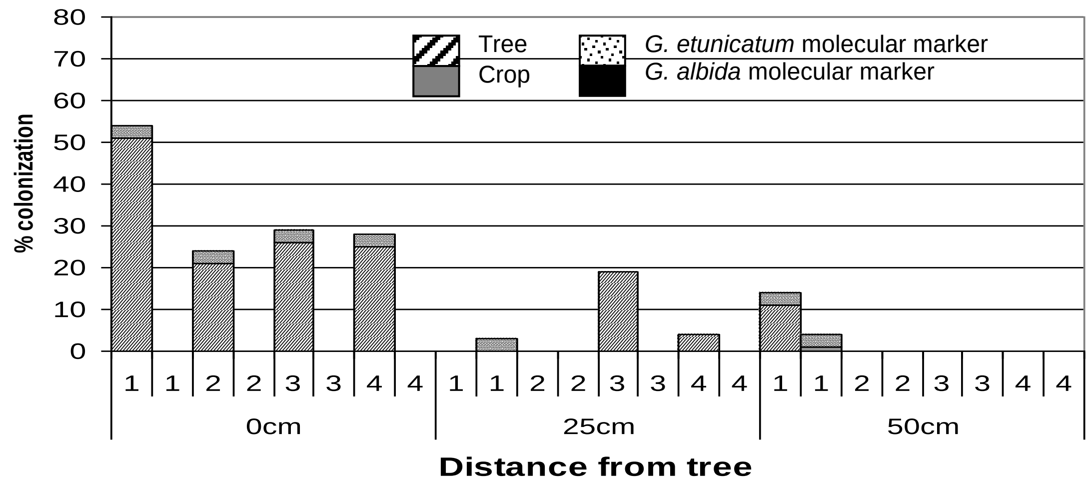

Figure 2b. Trees inoculated with *G. albida*

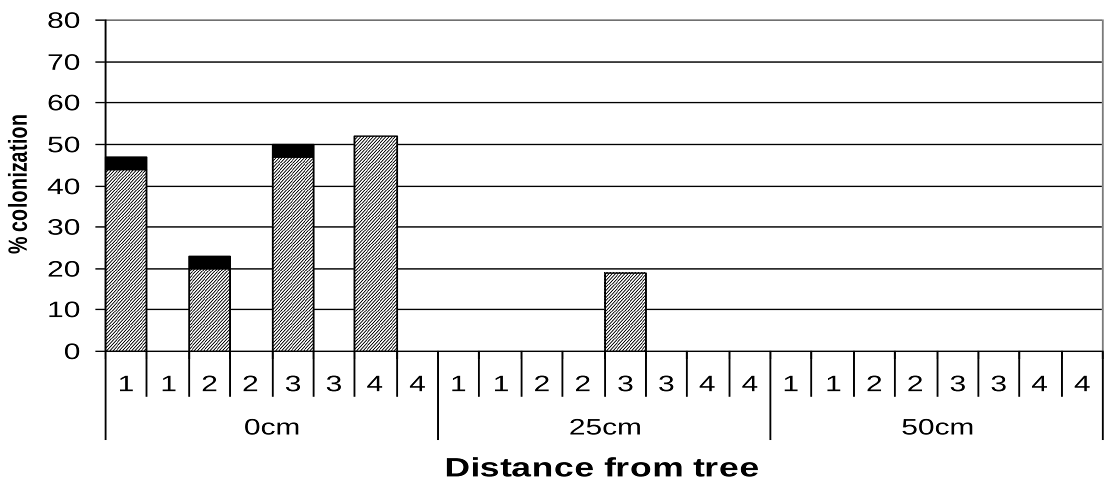

Figure 2c. Trees uninoculated

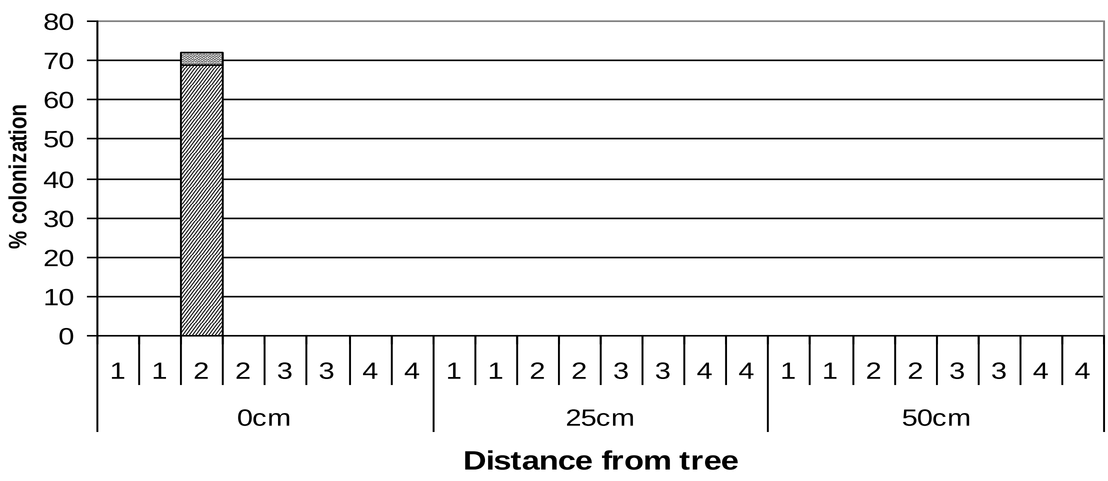

Figure 3a. Trees inoculated with *G. etunicatum*

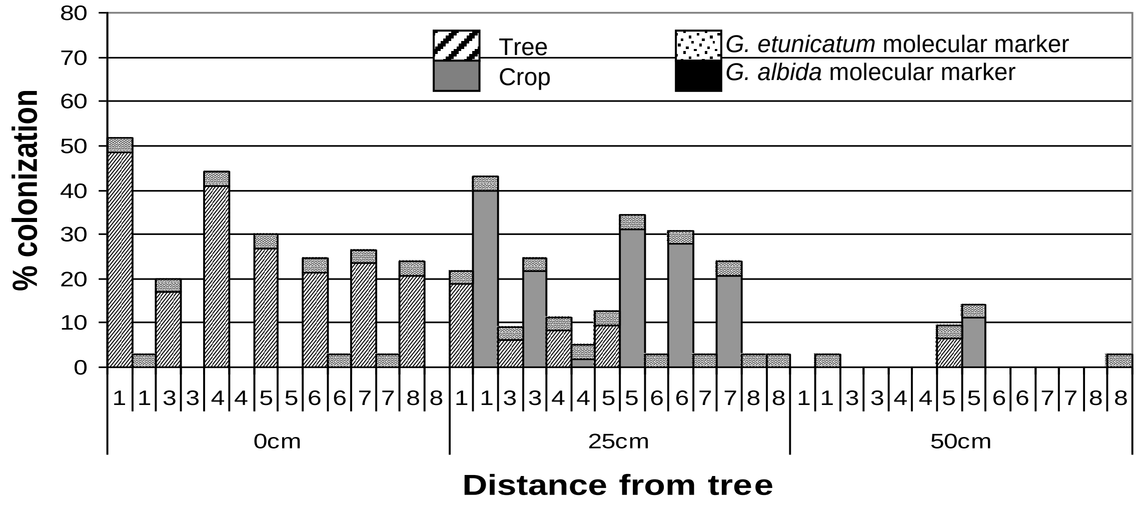

Figure 3b. Trees inoculated with *G. albida*

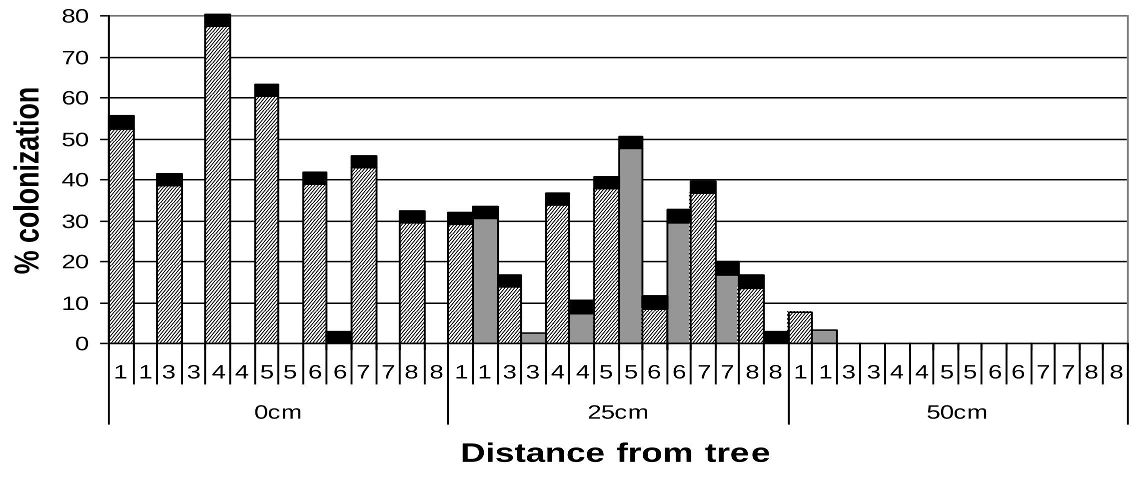

Figure 3c. Trees uninoculated

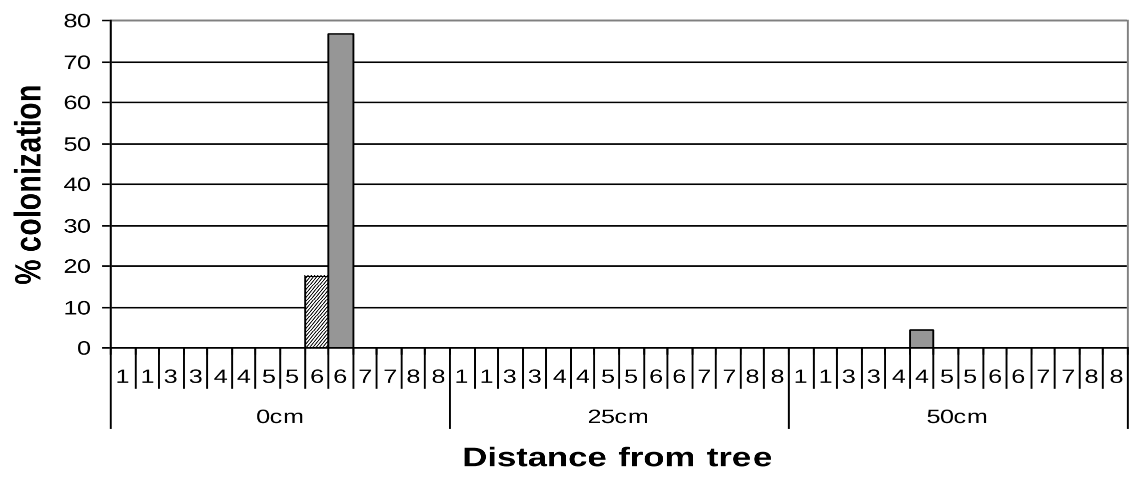

Figure 4a. Trees inoculated with *G. etunicatum*

Figure 4b. Trees inoculated with *G. albida*

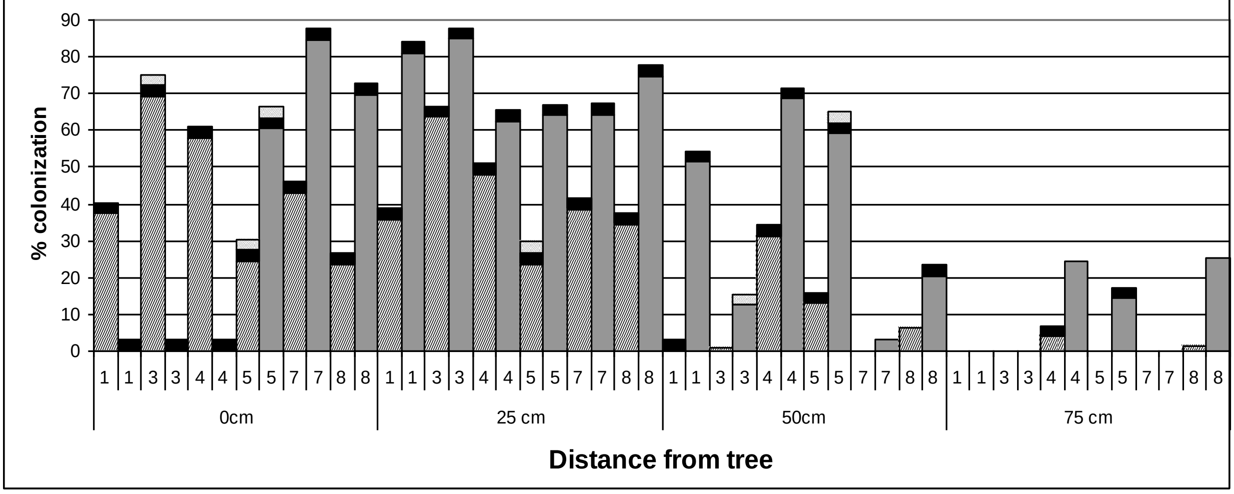

Figure 4c. Trees uninoculated