CENTRE FOR ECOLOGY AND HYDROLOGY
(NATURAL ENVIRONMENT RESEARCH COUNCIL)

CEH Project C01932
EU Project Contract number ICA4-2000-20037
Project acronym: SAFSYS

SYMBIONTS IN AGROFORESTRY SYSTEMS: WHAT ARE THE LONG-TERM IMPACTS OF INOCULATION ON GROWTH OF *CALLIANDRA CALOTHYRSUS* AND ITS INTERCROPS?

FINAL REPORT
December 2002 - December 2006

Partner 3: CEH
CEH Project number C01932

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December 2006

1. Objectives

The specific objectives of CEH Edinburgh (Partner 3) in the project were to:

1. Receive training from Partner 4 (SAC) in PCR, cloning and sequencing of AM fungi to initiate AM molecular studies at CEH. (**WP3: task 1**)

2. Obtain additional DNA sequences of the AM inoculants for inclusion in the project database. (**WP3: task 1**)

3. Characterise AM spore populations in soil from the experimental field sites in Kenya, Senegal and Zimbabwe. (**WP1: task 2**)

4. Assess AM infection using microscopy and molecular probes in root samples from the field experiments in Kenya. (**WP3: tasks 2+3**)

5. Maintain pot cultures of the 5 inoculant fungi at CEH, provide starter cultures when necessary, and deposit reference cultures of the 5 inoculant fungi with the International bank for the Glomeromycota (BEG). (**WP3: task 4**)

By 2004, it was apparent that specific primers developed for the inoculant fungi for use as molecular probes could not differentiate from the AM fungi occurring naturally in the field soils. As a result, the assessments of AM infection in root samples from the field experiments were curtailed and a glasshouse trough experiment was set up at CEH. The objectives of this experiment were to:

6. Examine more fundamental aspects of AM inoculant compatibility with tree and intercrop species, and the rate of spread and transfer of the inoculant fungi from tree to crop roots. (**WP3: tasks1+2+3**)

7. Develop methods for root sampling, extraction of fungal DNA from the roots and use of molecular probes. (**WP3: task 1**)

2. Activities

2.1. Molecular training at SAC (Partner 4)

Training in the molecular techniques used for AM fungi was provided to CEH staff from 17-19 April 2002. Among the techniques covered were; DNA extraction from AM spores, PCR amplification, transformation, cloning and screening of vector plasmids, purification and sequencing.

2.2. Cloning and sequencing of AM inoculants

After the training at SAC, protocols were adapted for use in the CEH laboratories, and laboratory registration and the necessary health and safety procedures were put in place. DNA extracts were made from multiple collections of spores extracted from pot cultures of *Gigaspora albida* 2 (BEG 173) and *Glomus etunicatum* 1 (BEG 176). Nested PCR products were inserted into *E.coli* plasmids and transformed. Resulting clones were isolated on selective agar plates and DNA from the bacterial colonies was amplified and purified for sequencing. Sequences were read manually from the gel images.

Rapid progress was made with molecular work at CEH during 2003 and resulted in a total of 37 sequences of *G. albida* 2 (BEG 173) and 8 of *G. etunicatum* 1 (BEG 176) being obtained and added to the project database, supplementing those already obtained by SAC. The additional sequences obtained for *G. albida* 2 were used to successfully modify the GA2 specific primer when this failed to amplify DNA from spore extracts and root samples from the glasshouse trough experiment.

2.3. Spore populations at field sites

Spore populations were examined in soils from the proposed field sites in Kenya, Zimbabwe and Senegal. Trap cultures using the soils were set up in the glasshouse at CEH in order to allow for sequential sampling and to fully explore AM fungi present in the soils. The results showed contrasting differences in numbers and diversity of viable spores in the soils. These differences appeared to vary according to local factors such as soil fertility, previous land use and seasonal fluctuations. Of particular relevance, was the occurrence of one of the inoculant fungi, *Glomus etunicatum*, in trap cultures using soil from the Muguga field site in Kenya as, in subsequent studies, it was found that the *G. etunicatum* 1 molecular probe could not differentiate from the AM fungi occurring naturally in the field soils.

Populations of AM fungi vary greatly between sites. These differences are not related to country of origin but to factors such as soil fertility and land use. These results simply emphasize the findings of previous studies that, before any inoculation is undertaken, the soil chemistry and microbial activity of the planting site should be thoroughly characterized in order to gauge the potential benefits of inoculation.

2.4. AM infection of root samples

High levels of infection were found in both inoculated and uninoculated *Calliandra* seedlings from the nursery. As none of the AM inoculants were known to form intraradical spores, the widespread occurrence of intraradical spores in the roots indicated that naturally occurring AM fungi were dominant. However, there were fewer intraradical spores present in inoculated seedling roots, which suggested that the inoculant fungi may have established on these roots. High levels of AM infection and intraradical spores were also found in crop roots sampled from inoculated plots in the field experiment.

Subsequent molecular screening of these root samples by SAC showed that the *Glomus etunicatum* 1 specific primer was amplified in all samples. As spores of this species were found in soils from Muguga, it is possible that the specific primer could not differentiate *Glomus etunicatum* 1 from the AM fungi occurring naturally in the field soils

2.5. Glasshouse trough experiment

By 2004, it was apparent that the assessments of AM infection in root samples from the field sites should be curtailed until a more specific primer could be developed for the *Glomus etunicatum* 1 isolate. It was therefore decided to conduct a glasshouse trough experiment at CEH which would examine more fundamental aspects of AM inoculant spread from tree to crop roots under controlled conditions. The objectives of this experiment were to:

1. Examine the compatibility of AM inoculants with tree and crop species and their ability to promote growth.

2. Determine the rate of spread and transfer of the inoculant fungi from tree to crop roots.

3. Develop methods for root sampling, extraction of fungal DNA from the roots and use of molecular probes.

Seedlings of *Calliandra calothyrsus* (Flores Ex. Maseno) were inoculated with AM inoculants *Glomus etunicatum* 1 (BEG176), *Gigaspora albida* 2 (BEG173) or uninoculated. All seedlings were inoculated with Rhizobial strains known to be effective for *C. calothyrsus*. Nine weeks after AM inoculation, seedlings were transplanted to troughs with inoculation treatments replicated in 8 randomised blocks. Growth of the trees was monitored by measuring stem diameter. During 2004-5, crops were sown 15, 28 and 64 weeks after inoculation at 25, 50 and 75 cm from the tree. At crop harvest, soil cores were removed at each distance so that tree and crop root samples could be obtained for mycorrhizal assessment. Presence or absence of the inoculant fungi was determined using the molecular probes and levels of mycorrhizal infection were assessed by microscopic examination of stained roots.

Effects of inoculation on tree growth are shown in Figure 1. Inoculated plants were significantly ($P<0.001$) larger than uninoculated plants even before transplanting to the troughs and remained so throughout the course of the experiment. The graph also indicates a reduction in the growth rate of inoculated trees after about 40 weeks, which was attributed to these much larger trees becoming increasingly pot-bound.

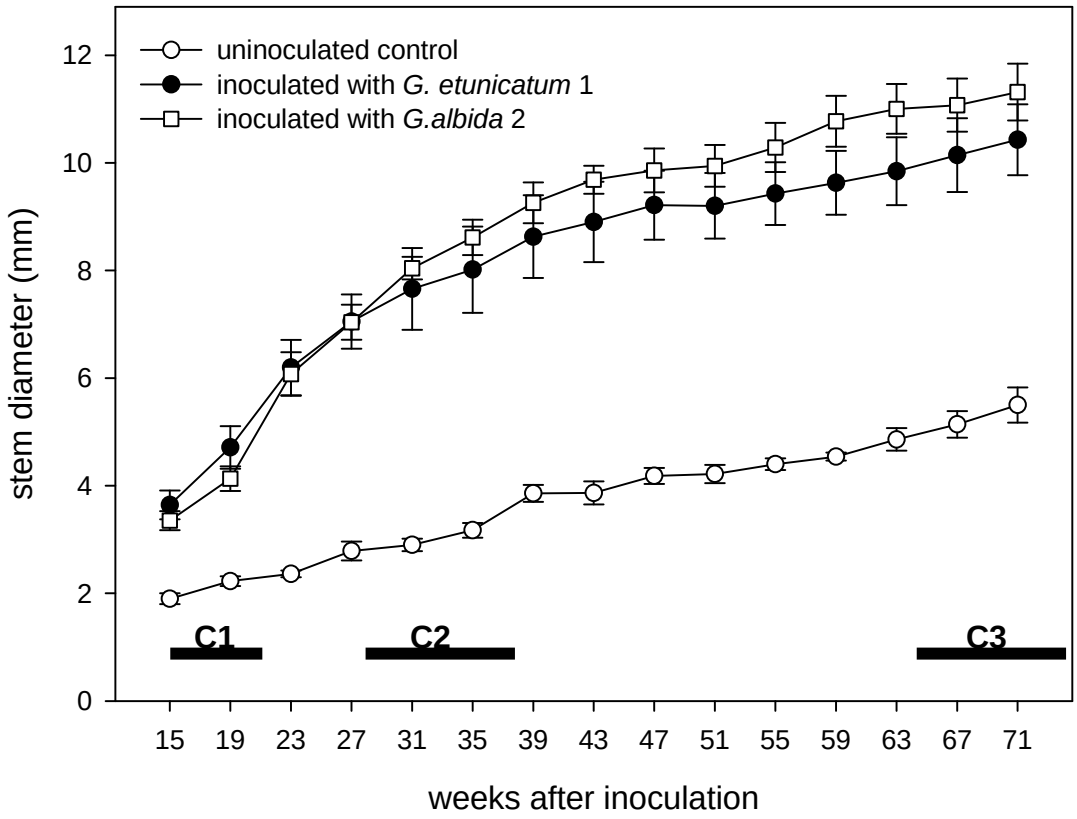
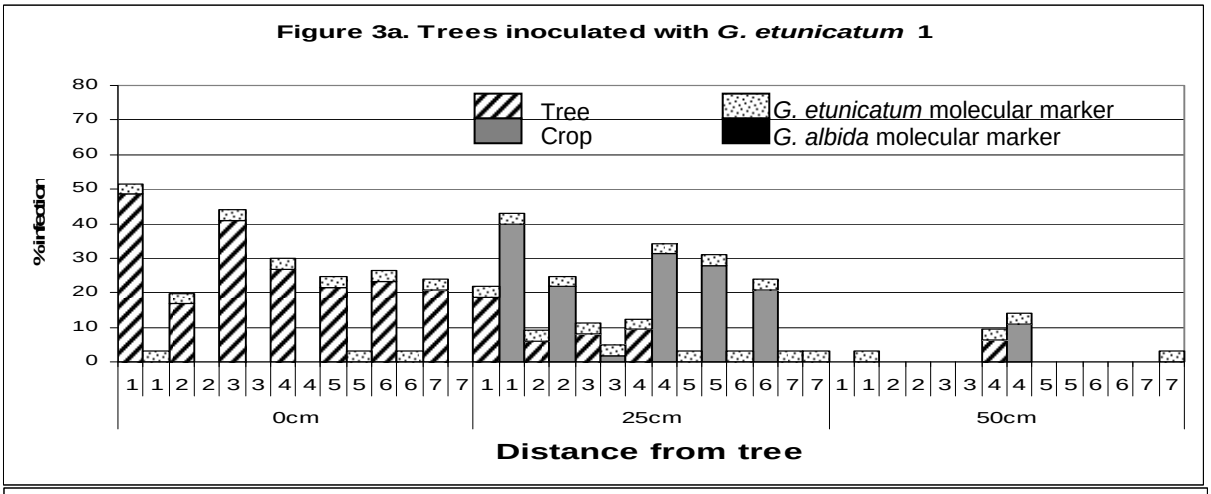
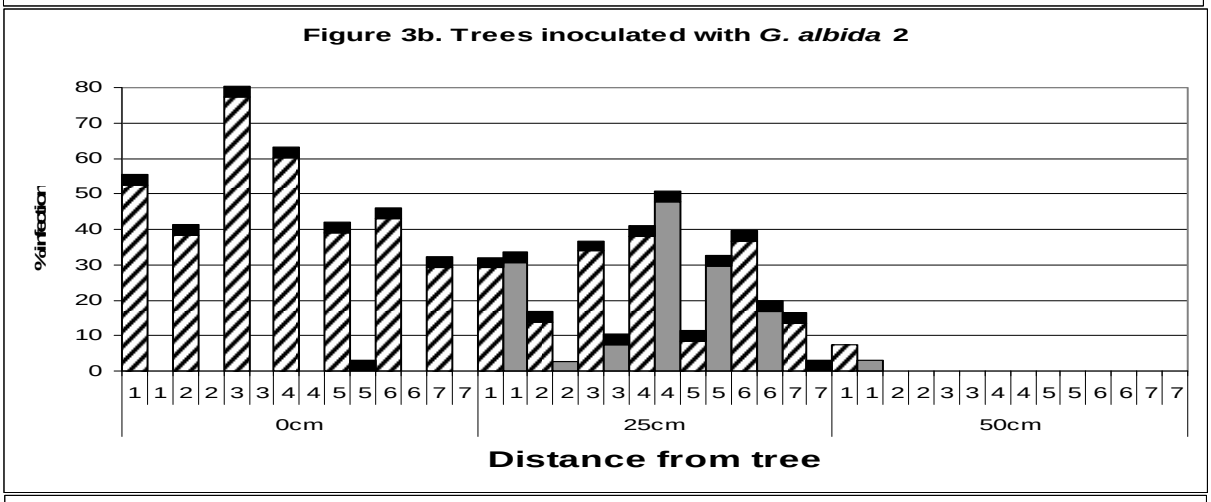


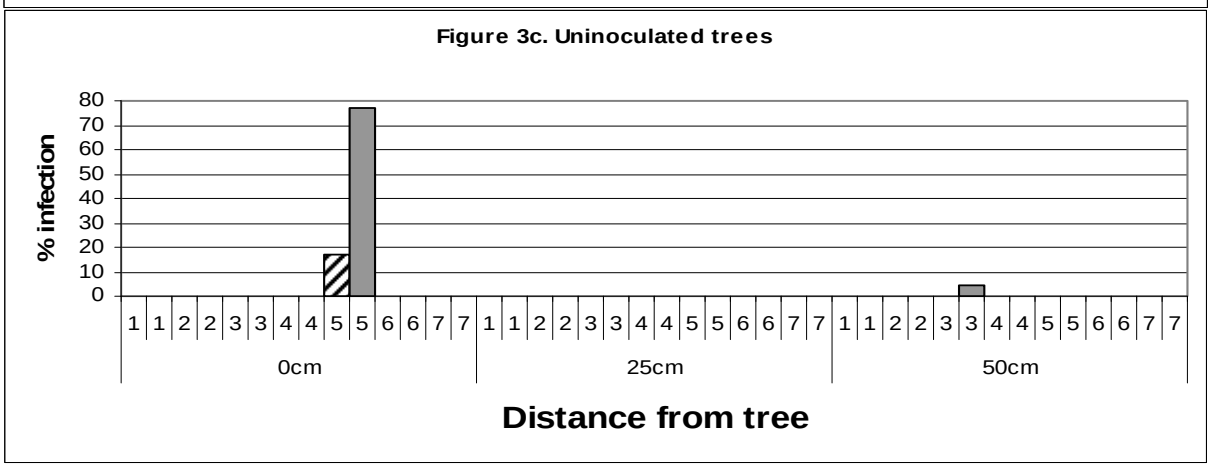
Figure 1. Stem diameter of inoculated and uninoculated *Calliandra calothyrsus* trees in a glasshouse trough experiment during 2004-5 (error bars = ±SE; horizontal bars indicate cropping periods C1-3)

Results from all the crop harvests showed that growth of the crop was reduced by competition with the tree. In the first two cropping periods, crop growth was significantly (P<0.001) better with increasing distance from the tree. In the third cropping period, trees were shoot pruned to reduce below ground competition. Although this resulted in better growth of the crop across all treatments and reduced the intense competition near the tree, crop growth was still found to be significantly (P<0.001) better in uninoculated troughs where the trees were much smaller.

By the time of the second crop harvest, roots of most trees had extended well over 50 cm from the tree with those of the larger trees reaching 75 cm. However, molecular detection of the inoculant fungi and levels of mycorrhizal infection showed that the inoculant fungi were established on the tree roots at 25 cm, but not at 50 cm from the tree (Figures 3a-c). When present on the tree roots at 25 cm, the fungi had successfully spread to the crop roots. Because of intense competition with the tree, few crop roots, and therefore little mycorrhizal infection were found near the tree at 0 cm. The graph shows that the molecular markers consistently differentiated between the 2 inoculant fungi, and indicates that cross contamination of fungi between inoculated troughs had not occurred at this stage. The graph also suggests, perhaps not surprisingly, that the molecular markers were more sensitive than microscopic observation, as there are several samples where the inoculant fungi were detected, but no mycorrhizal infection was recorded.

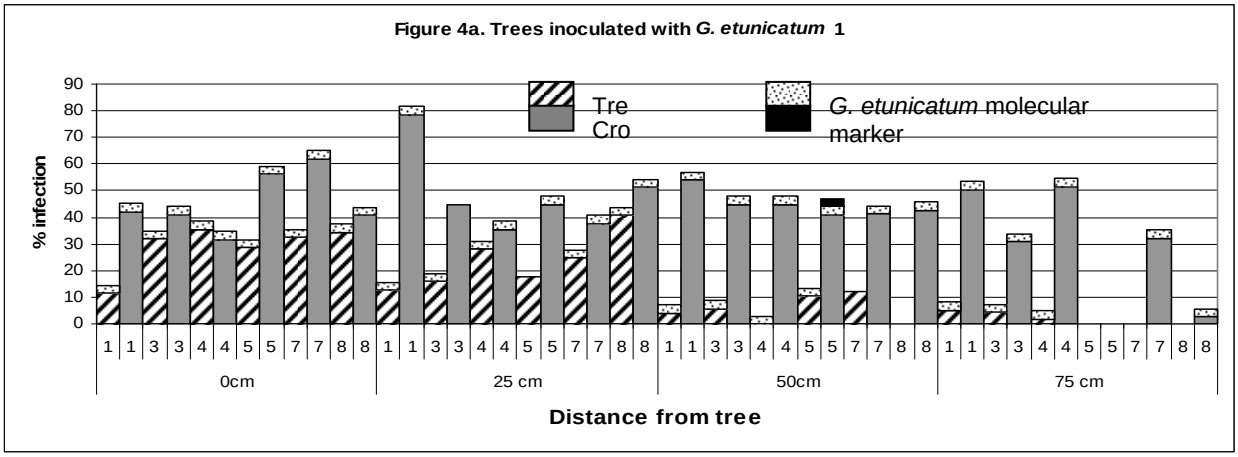


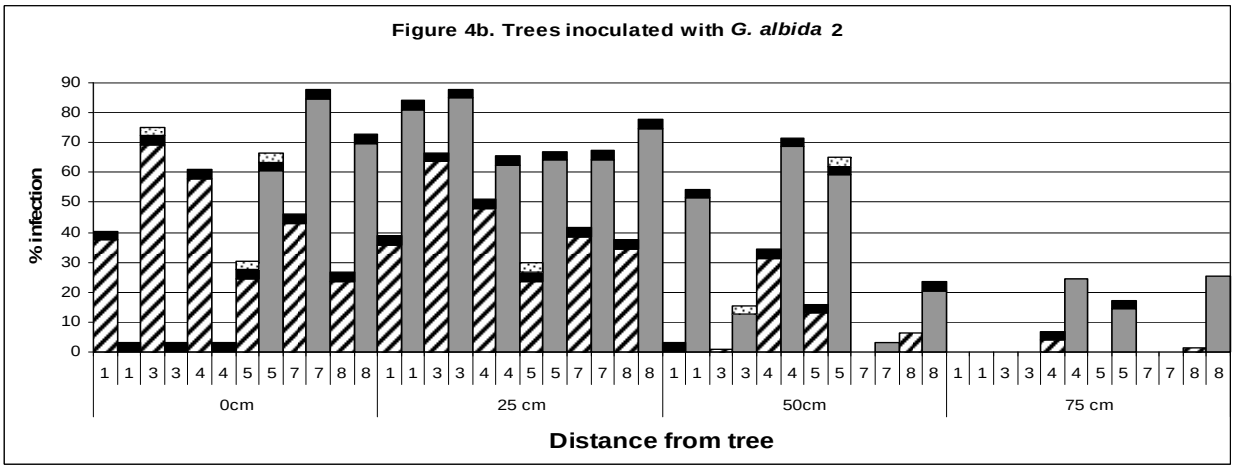


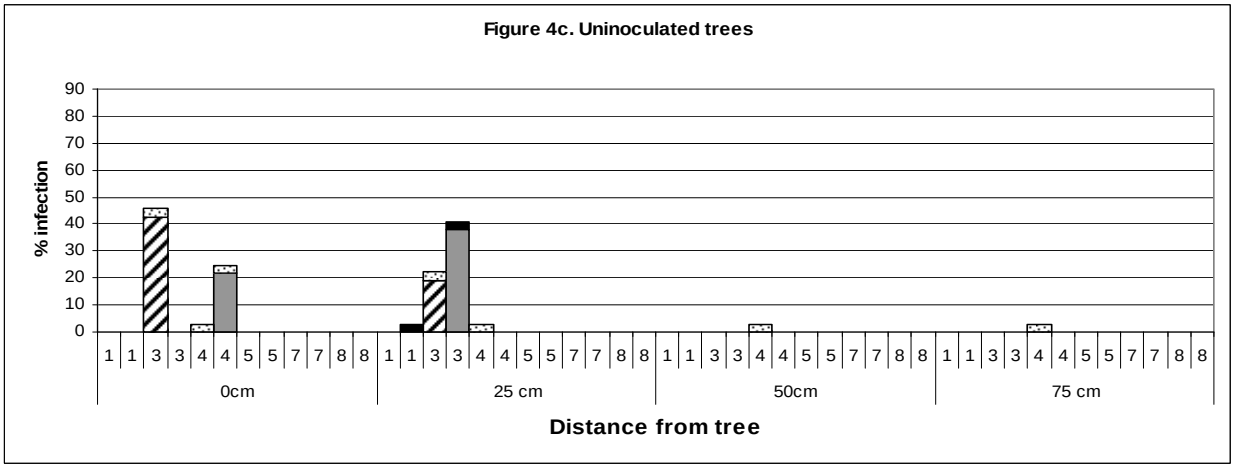


Figures 3a-c. Extent of mycorrhizal infection (% root length) determined microscopically, and origin (*G. etunicatum* 1, *G. albida* 2 or other) of mycorrhizal fungus determined by molecular methods, on tree *(C. calothyrsus)* and crop *(Z. mays)* roots growing together in troughs. Samples collected in November 2004. Trees were previously inoculated with *G. etunicatum* 1 (Fig. 3a), *G. albida* 2 (Fig. 3b) or not inoculated (Fig. 3c). 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.

By the time of the third crop harvest, tree roots had extended throughout the troughs in all treatments and the larger inoculated trees were pot bound. Molecular detection of the inoculant fungi and levels of mycorrhizal infection showed that *G. etunicatum* 1 had established on the tree roots at 75 cm and had successfully spread to the crop roots (Figures 4a-c). Spread of *G. albida* 2 appeared to be slower and its presence, and spread to crop roots, at 75 cm was more sporadic. Because of the shoot pruning carried out prior to sowing of the crop, competition with the tree was less intense, and crop roots were found near the tree at 0 cm and had become infected. The graphs also show that cross contamination of the inoculant fungi between inoculated and uninoculated troughs had now occurred, with *G. etunicatum* 1 responsible for most of the cross contamination. The graphs again show that the molecular markers were more sensitive than microscopic examination, as there are several samples where the inoculant fungi were detected, but no mycorrhizal infection was recorded.







Figures 4a-c. Extent of mycorrhizal infection (% root length) determined microscopically, and origin (*G. etunicatum* 1, *G. albida* 2 or other) of mycorrhizal fungus determined by molecular methods, on tree *(C. calothyrsus)* and crop *(Z. mays)* roots growing together in troughs. Samples collected in August 2005. Trees were previously inoculated with *G. etunicatum* 1 (Fig. 4a), *G. albida* 2 (Fig. 4b) or not inoculated (Fig. 4c). 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 results of the glasshouse trough experiment showed that –

1. Both the AM inoculants tested greatly benefited growth of *Calliandra calothyrsus* and transferred readily to crop roots. Potential growth benefits to the crop of mycorrhizal infection by the inoculant fungi could not be examined because of intense competition with the tree in the restricted soil volume of the troughs.

2. The rate of spread of the inoculant fungi on the tree roots was slower than expected and was much slower than the growth of the roots themselves. This suggests it may take years before inoculants benefit growth of crops sown several metres from the tree.

3. Shoot pruning reduced competition between tree and crop, and did not appear to impair the viability of the inoculant fungi. This suggests that normal tree management procedures will not damage the activity of AM inoculum in agroforestry systems.

4. Methods for root sampling, extraction of fungal DNA from roots and use of molecular probes were developed and successfully applied. Rigorous, aseptic root sampling protocols are needed for this kind of work.

5. Microscopic quantification of mycorrhizal infection and the use of molecular probes to identify specific fungi within roots complemented 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.

2.6. Culturing and accreditation of AM inoculants

During the course of the project, pot cultures of 18 AM isolates were maintained in the CEH glasshouse. Six *Glomus* spp. isolates were supplied to Partner 4 (SAC) to aid in the development of a more specific primer for *Glomus etunicatum* 1.

Cultures of the 5 selected AM inoculants were deposited with the BEG international mycorrhizal culture collection, and were assigned the following accession numbers:-
BEG 172: *Gigaspora albida* 1b
BEG 173: *Gigaspora albida* 2
BEG 174: *Scutellospora calospora* 2
BEG 175: *Scutellospora verrucosa* 2c
BEG 176: *Glomus etunicatum* 1
Full details of these isolates are available on the BEG website: www.kent.ac.uk/bio/beg/

3. Dissemmination

1. A paper entitled 'Do trees and crops share mycorrhizas in agroforestry systems?' was presented at the British Ecological Society conference in September 2005.

2. Manuscript submitted to Plant and Soil journal:
Ingleby K, Wilson J, Munro R C and Cavers S. Mycorrhizas in agroforestry: sharing and spread of arbuscular mycorrhizal fungi between trees and crops – complementary use of molecular and microscopic approaches.

4. Project outcomes

1. CEH received training from Partner 4 (SAC) in 2002, and initiated molecular work in CEH laboratories which provided valuable sequence data during 2003.

2. Molecular skills at CEH were extended in 2004 and 2005 with the development of methods for the use of the fungal specific primers as molecular probes.

3. AM spore populations in soil from the experimental field sites in Kenya, Senegal and Zimbabwe varied according to soil fertility, land use and season: one of the inoculant fungi, *Glomus etunicatum*, was present at the Kenyan field site.

4. Root infection assessments of field samples suggested that the inoculant fungi may have established on the trees, but that naturally occurring AM fungi were dominant.

5. *Glomus etunicatum* 1 molecular probe could not differentiate from naturally occurring AM fungi in the field: further refinement of the fungal specific primers was needed to improve its specificity.

6. A glasshouse trough experiment conducted in 2004 -2005 showed that both the AM inoculants tested greatly benefited growth of *Calliandra calothyrsus* and transferred readily to crop roots.

7. The experiment showed that the rate of spread of the inoculant fungi on the tree roots was slower than expected and that it may take years before inoculants benefit growth of crops sown several metres from the tree.

8. The experiment showed that shoot pruning reduced competition between tree and crop, and did not appear to impair the viability of the inoculant fungi: this suggests that normal tree management procedures will not damage the activity of AM inoculum in agroforestry systems.

9. Results from the experiment were presented at the British Ecological Society conference in September 2005. A paper has also been submitted to Plant and Soil.

10. Pot cultures of the 5 inoculant fungi were maintained in the CEH glasshouse: reference cultures were deposited with BEG, validated and given accession numbers.